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MASS TAGGING OF BLACK FLIES (Diptera : Simuliidae) WITH RADIOPHOSPHORUS[1]

BY F. J. H. FREDEEN[2], J. W. T. SPINKS[3], J. R. ANDERSON[4], A. P. ARNASON[5], AND J. G. REMPEL[6]

Abstract

A new method for tagging large numbers of black flies (*Simulium* spp.) for flight range investigations is described. Radioactive adults that were readily detected with a Geiger counter were reared from larvae that had been kept for 24 hr. in a very dilute solution of radioactive phosphorus (0.2 μc. per ml.) and then returned to nonradioactive water to complete their development. The treatment did not visibly harm larvae or the adults that emerged from them. Larvae were reared in small containers of water circulated and aerated with air jets in the laboratory, and with a simple arrangement of stream-driven paddle wheels in the field. By this method, tagged larvae, pupae, and adults were produced which could be readily detected with a Geiger counter. In field tests, tagged larvae and pupae were found as far as 520 yd. downstream from the point of release. Tagged adults were taken in cages placed over radioactive larvae and pupae in the stream, but only one was recaptured in the open, and this only 100 yd. from the stream. Failure to capture more tagged adults was believed due mainly to inadequate collecting methods. A short treatment period is desirable when tagging large populations of black-fly larvae in the field because other methods, such as rearing in a small container for a long period of time, or treating the stream with a radioactive isotope, are impractical. This method would be applicable in studies on dispersal and predation of immature and adult stages of other insects of streams and ponds.

Introduction

Radioactive tracers have been widely applied in entomological research in recent years (Jenkins and Hassett (8)). One of the most recent applications is in studies of dispersal of blood-sucking insects (Bugher and Taylor (2); Hassett and Jenkins (5, 6); Jenkins (7); Thurman and Husbands (10); Yates *et al.* (11)). These have all been made with mosquitoes; no attempts to utilize radioactive tags in studies of black-fly dispersal have been reported. The only report of the successful use of a tag in studies of black-fly dispersal is that by Dalmat (4), who marked a limited population by dusting adults with an aniline dye.

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In recent studies of the flight habits of *Simulium arcticum* Mall. in Saskatchewan and of black flies in northern Canada, the need for a simple, quick, and reliable method of tagging large populations has become apparent. The present paper describes a method developed in 1950 and 1951 for the use of radiophosphorus to tag black flies. The immature stages were selected for tagging studies because they were relatively plentiful and were easily collected in comparison with the adults. Initial laboratory tests indicated that larvae readily absorbed radiophosphorus from water, but because they occurred only in rapidly flowing water, the methods used for pond-inhabiting insects, such as mosquitoes, were not directly applicable. The method developed is well suited for tagging large populations of larvae in a short period and may also be applied, with slight modifications, to studies of other stream-inhabiting organisms. The method also satisfies each of the following requirements for an ideal marking procedure: (*a*) ease of application to large populations; (*b*) minimal effect on larval and pupal development; (*c*) ease of recognition of the tagged black flies without necessarily killing them; (*d*) persistence of the marker throughout the life of the black fly; (*e*) freedom from harmful biological effects; (*f*) a radioactive tag with a half life sufficiently short to minimize contamination problems; and (*g*) ready availability.

Laboratory Experiments

Laboratory experiments were conducted to determine the feasibility of using radiophosphorus for tagging black flies and to provide information required to undertake mass radioactivation in the field. Individual tests were designed to determine the effect on the ultimate radioactivity of the adult of (*a*) concentration of P^{32}; (*b*) duration of the radioactivation period; (*c*) size, age, and concentration of larvae; and (*d*) the rate of loss of radioactivity after radioactivation.

In preliminary experiments, black-fly larvae were reared in 5-liter battery jars and in 1-liter berzelius beakers containing water circulated by compressed air from a small compressor unit. The compressed air, entering at the bottom of a 6 in. section of 1 in. diameter glass tubing immersed in the water, produced a vigorous circulation. Larvae of *Simulium venustum* Say and *S. vittatum* Zett. attached themselves to the inside wall of this tube where the water current was strongest (Fig. 1).

Water used in the experiments was obtained from the stream in which the larvae were collected. The temperature during the experiments was approximately 77° F. A cage of fine netting was placed over each aquarium to prevent adults from escaping upon emergence (Fig. 2).

Radiophosphorus, as $P^{32}O_4$ in dilute hydrochloric acid, was obtained from Chalk River, Ontario, for these experiments. P^{32} is a beta emitter (average energy 0.69 Mev.) with a half life of 14.3 days. Radioactivity was measured in the laboratory with an end-window Geiger counter and a scale of 64 scaler. The counter was calibrated by means of a Radium D + E standard.

PLATE I

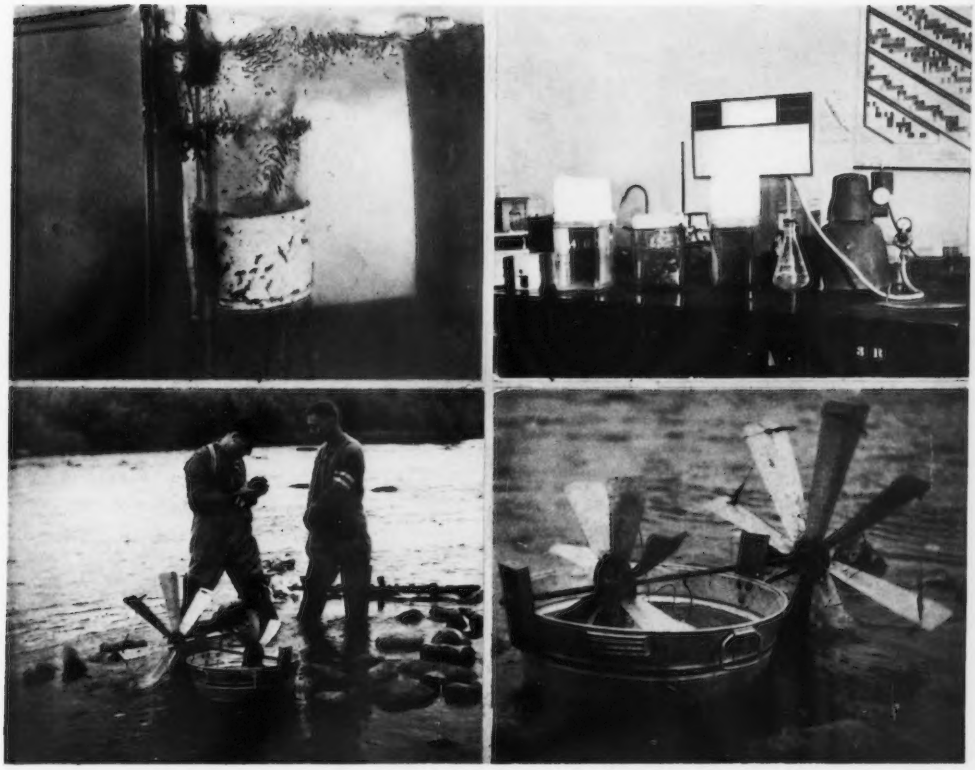

FIG. 1 (Upper left). Black-fly larvae attached to wall of aquarium in water circulated by compressed air.

FIG. 2 (Upper right). Laboratory equipment: air compressor, wash bottle, and aquaria for larvae. The Geiger counter and counter box used in field work are also shown.

FIG. 3 (Lower left). Apparatus used in field experiments on the Torch River, 1950, for rendering black-fly larvae radioactive.

FIG. 4 (Lower right). Apparatus used for rearing black-fly larvae in radioactive solutions in the field. The solution is circulated and aerated by a small paddle wheel in the tub driven by a larger paddle wheel in the stream.

In the first three tests mortality of larvae was high. This was attributed to a lack of suitable food in the water. In subsequent laboratory and field tests, black flies were reared from eggs to adults by the addition of a small amount of yeast[7] to the water.

Test 1

The object of the first laboratory experiment was to determine whether black-fly larvae could be tagged by rearing them in solutions containing a small amount of radioactive phosphate ion. One thousand larvae were placed in each of two aquaria: a 2-liter aquarium with a concentration of 0.019 μc. of P^{32} per ml., and a 6-liter one with 0.005 μc. per ml. Larvae were left in the solutions from $1\frac{1}{2}$ to 71 hr., washed for a few seconds in 1% phosphate solution to remove adhering P^{32}, rinsed in distilled water, and dried on filter paper. For larvae kept in the weaker solution for $1\frac{1}{2}$ hr. the radioactivity ranged from 89 to 3680 disintegrations per minute per larva, depending upon the size; at the end of 19 hr. the average radioactivity was 1450 disintegrations per minute, and this increased gradually to 1695 for the maximum exposure period. Larvae from the more concentrated solution possessed about the same amounts of radioactivity. The larvae absorbed 85% of the total radioactivity during the first 19 hr. Adults were not obtained from these larvae, but two chironomid adults that emerged from the aquaria had radioactivities of 1900 and 5200 disintegrations per minute. The rearing method was not entirely satisfactory because considerable mortality occurred in both treated and untreated aquaria. Nevertheless, the results showed that black-fly larvae absorbed considerable amounts of P^{32} from dilute solutions in a short period, and that further tests to develop a method for tagging large numbers of larvae were warranted.

Test 2

The purpose of the second test was to determine the rate of uptake and the subsequent rate of loss of radioactivity from larvae that were reared in radioactive solutions and later placed in nonradioactive media. Thirty-five larvae were placed in each of three 300-ml. berzelius beakers containing 0.2, 0.02, and 0.002 μc. of P^{32} per ml. respectively. Table I indicates that the radioactivity of larvae increased with the concentration of P^{32} in the solution and with the exposure time.

There was considerable depletion of the radioactivity of the solutions during the experiment, mainly as a result of absorption by larvae but also partly as a result of natural decay of the P^{32}.

Larvae that had been exposed for 20 hr. to the radioactive solution and then placed in a nonradioactive solution for 24 hr. retained approximately 60% of their radioactivity.

[7] *Yeast was cultured by mixing 9 gm. of dry yeast (Fleischman's) with 9 gm. of sugar in 500 cc. of water and allowing it to stand for several hours. A small amount (0.2 to 3.0 mgm. per cc.) of this culture was added to each aquarium.*

TABLE I

UPTAKE OF P^{32} BY NEARLY MATURE BLACK-FLY LARVAE

Concentration of P^{32} in solution (μc. P^{32} per ml.)	Exposure time (hr.)	Average radioactivity of larvae (disintegrations per min.)
0.2	1	2350
0.02	1	870
0.002	1	770
0.2	20	890,000
0.02	20	9850
0.002	20	2740

Test 3

In the third test, 300 larvae were placed in each of two 5-liter aquaria containing 0.02 μc. of P^{32} per ml. In 10 to 16 days 33 adults emerged. These had an average radioactivity of about 5000 disintegrations per minute.

Test 4

The purpose of the fourth test was to determine whether radioactive adults could be obtained when larvae were kept for a short period in a solution containing P^{32} and then reared in a nonradioactive medium; also to determine the relationship between larval size and the amount of radioactivity absorbed.

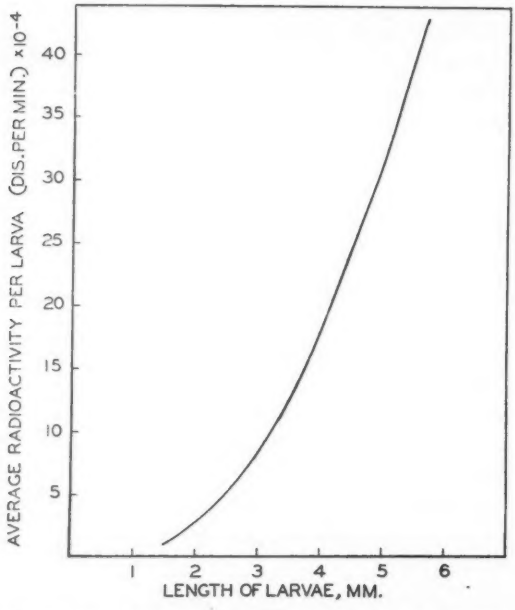

FIG. 5. Relationship between the length of a larva and the total amount of P^{32} absorbed during a 23 hr. immersion in a solution containing 0.245μc. of P^{32} per ml.

Approximately 200 larvae were placed in 900 ml. of solution containing 0.245 μc. of P^{32} per ml. Twenty-three hours later about half of the larvae were removed for measurement of radioactivity, and the remainder were transferred to a nonradioactive 5-liter aquarium. A logarithmic plot of Fig. 5 indicates that the uptake of P^{32} by the larvae was proportional to their volume.

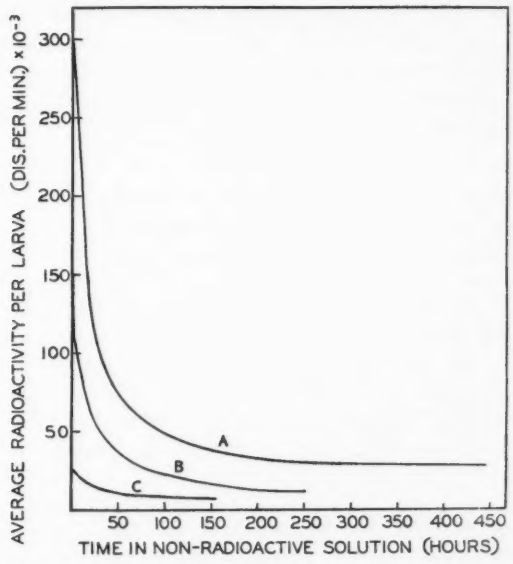

FIG. 6. Rate of loss of radioactivity from larvae kept in a nonradioactive solution after a 23 hr. immersion in a solution containing 0.245μc. of P^{32} per ml.
A, 4.1 to 6 mm. larvae; B, 2.5 to 4 mm. larvae; C, 1 to 2.4 mm. larvae.

Larvae were removed from the nonradioactive aquarium at approximately two-day intervals and their radioactivity was measured. The results (Fig. 6) indicate that the loss of radioactivity was greatest during the first two days, and that the rate of loss decreased with time. However, larvae were still

TABLE II

RADIOACTIVITY OF BLACK-FLY ADULTS REARED FROM LARVAE IMMERSED FOR 23 HR. IN A SOLUTION CONTAINING 0.245 μC. OF P^{32} PER ML., AND THEN PLACED IN A NONRADIOACTIVE MEDIUM

Time in inactive medium (days)	No. of flies	Average radioactivity of adults at time of emergence (dis. per min.) $\times 10^{-4}$
Up to 9	5	7.0
10	3	3.7
14 to 17	11	3.1
18 to 19	11	2.5
20 to 24	2	1.6

highly radioactive at the end of 24 days. The mortality of these larvae was approximately the same as that in a check group. Thirty-two highly radioactive adults emerged from the larvae (Table II). Adults lost less than 20% of their radioactivity in the first 24 hr.

Test 5

In the fifth test, a six hour exposure was tried. The radioactivity taken up was about one sixth that in the 24 hr. exposure, and the radioactivity retained (approximately 3000 disintegrations per minute) was about one tenth that after the 24 hr. exposure, thus indicating that an exposure period longer than six hours was desirable.

Test 6

A test was made to determine the effect of the concentration of larvae on the radioactivity taken up. An initial radioactivity of 0.2 μc. per ml. was established in the rearing solution in one group and 0.05 μc. in another; a 24-hr. exposure was used.

The results (Table III and Fig. 7) indicate that the most highly radioactive larvae were obtained in solutions containing the fewer larvae and the higher concentration of P^{32} per unit volume. The results also indicated that as many as two to four larvae per milliliter could be kept in a solution containing 0.2 μc. of P^{32} per ml. without excessive mortality, and that after a 24 hr. treatment the average radioactivity of such larvae was equivalent to 100,000 to 160,000 disintegrations per minute. In the series using 0.05 μc. per ml. a greater number of pupae were formed during treatment than in the 0.2 μc. per ml. solution, because the larvae were more mature. As a result of this pupation and also because of some mortality of larvae, particularly in the more heavily populated aquaria, the results are not comparable. However, in the series using 0.2 μc. per ml. a plot of radioactivity in the larvae versus the log of the number of larvae per unit volume was linear, indicating a convenient method for interpolation.

TABLE III

RADIOACTIVITY OF BLACK-FLY LARVAE AFTER A 24 HR. EXPOSURE TO 0.2 OR 0.05 μC. OF P^{32} PER ML.

Initial concentration of solution (μc. per ml.)	No. of larvae per ml.*	Av. radioactivity of larvae (dis. per min.)	Per cent pupation during test
0.2	6.45	46,500	14.0
0.2	6.05	61,600	9.6
0.2	3.09	111,000	26.2
0.2	1.89	169,000	19.8
0.2	0.65	251,000	9.5
0.05	3.80	18,800	30.6
0.05	1.78	25,800	33.0
0.05	1.38	33,200	38.8
0.05	0.92	37,600	33.8

* *Including those that pupated during the treatment.*

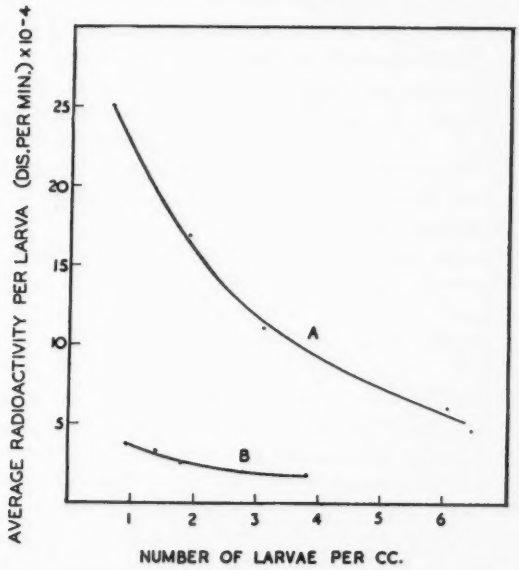

FIG. 7. Effect of concentration of larvae on the amount of P^{32} absorbed during a 24 hr. exposure in a solution containing:
A, 0.2 μc. and B, 0.05 μc., of P^{32} per ml.

Test 7

Finally, pupae were immersed in a solution containing 0.01 μc. per ml. for one to three days and then reared to adults in vials. These adults had little or no radioactivity.

Field Experiments

The results from laboratory tests suggested that tagged adults could be produced in the field by transferring larvae to a small volume of solution containing 0.2 μc. of P^{32} per ml., and after 24 hr. returning them to a stream. The principal modification required for field work was a means of maintaining movement and aeration of water in the container. This was accomplished by the stream-driven apparatus described below. Field tests in 1950 were made on the Torch River and in 1951 on the Battle River, both in central Saskatchewan.

The Torch River (Fig. 3) near White Fox, Saskatchewan, where the experiments were conducted in 1950, averages 100 ft. in width, and flows for more than a mile in a series of rapids, forming an excellent site for black flies. Larvae and pupae of *Simulium luggeri* N. & M. and of *S. venustum* were found on rocks and grass leaves in swift water. On July 18 and 19 the discharge was estimated to be about 300 c.f.s., and the maximum and minimum water temperatures were 71° F. and 63° F. respectively.

Black-fly larvae were activated in two round 50 liter galvanized iron tubs filled to a depth of 8 in. with river water (Fig. 4).

The water in each tub was kept in motion by means of a 14 in. galvanized iron paddle wheel on a shaft driven by a 24 in. paddle wheel dipping in the stream. The shaft was mounted on hardwood bearings across the top of the tub. It was necessary to coat the tub and paddles with paraffin wax to prevent adsorption of P^{32}. Yeast was added to provide food for the larvae.

The source of radioactivity was 100 mc. of $P^{32}O_4$ in dilute hydrochloric acid obtained from Chalk River, Ontario. The radioactivity of the black flies was detected and quantitatively determined in the field with a portable beta-gamma count rate meter, the Geiger probe being built into a counter box[3]. Field counts were checked against a laboratory Geiger-Müller counter in Saskatoon.

Black-fly larvae were readily collected from the river. Vegetation with attached larvae was collected, and the larvae were washed off the vegetation and strained through netting. Care was taken to remove all traces of vegetation that might absorb P^{32} from the solution. After the total volume of the larvae was measured they were placed in the rearing tub. An aliquot volume of larvae from one test was preserved to determine the number per milliliter, and this was used as a base in determining the number of larvae used in each treatment. It was also possible to determine the number of larvae in a treatment by dividing the average amount of P^{32} absorbed by a larva into the total amount of P^{32} removed from the solution. Discrepancies were due mainly to loss of larvae during treatments, because larvae quickly lost P^{32} after death.

After the introduction of larvae into a rearing tub, sufficient P^{32} solution was added to produce radioactivity of approximately 0.2 μc. per ml. Samples of the radioactive solutions were taken for measurement of radioactivity, both before and after each group of larvae was treated. In the field in 1950, these were evaporated in an aluminum dish and the radioactivity was determined with the portable survey meter. Later these were taken to Saskatoon for more accurate analysis. In 1951, the radioactivity of the solution was determined directly by placing the Geiger probe in a fixed position just above a small dish containing a known volume of the solution.

In the 1950 tests, eight groups of larvae were treated and released into the Torch River. The first four treatments were made in unwaxed, galvanized iron tubs, and apparently there was some loss of radioactivity by adsorption. Subsequent treatments were made in waxed tubs. The great reduction of radioactivity in the solutions, due to the absorption of P^{32} by the larvae, was not fully appreciated at first. As a result, the solution strengths during tests 2 and 3 were lower than desired.

Before the larvae were returned to the stream, 10 or more were removed from each group to determine their radioactivity. In addition, larvae were removed from the fifth test at five- to six-hour intervals to determine the rate of uptake of radioactivity.

Larvae in the rearing tubs were allowed to attach themselves to weighted bottles. These had wire handles projecting above the surface of the solution.

[3] *Nuclear Instrument Company, Model 2610A.*

At the end of each treatment period the bottles with attached larvae were carried out into the stream and the larvae removed by agitating the bottles in the water. The larvae from all the treatments were released at the same location in the stream. Tree branches were anchored across the rapids for a total distance of 100 yd. downstream from the point of release, in an effort to concentrate all the treated larvae in a relatively small area. Radioactive larvae were readily located on the leaves on these branches. It was necessary, however, to bring the leaves with attached larvae above the surface of the water for the radiation to be detected with the Geiger counter.

Between July 18 and Aug. 10, approximately 100 collections of adults were made with sweep nets from livestock and from vegetation along the river shore. The herds of livestock from which collections of adults were taken were pastured 0.5 to 4.5 miles from the rapids, and in sectors of territory towards which the prevailing winds blew from the rapids. The last collections of immature stages from the stream and of adults from livestock and from vegetation along the river shore were made on Aug. 22 and 23. Light traps were used, and two were set on the river shore at the experimental site. These were operated nightly from July 19 to Aug. 9.

The second field test was made in July and August, 1951, on the Battle River in west-central Saskatchewan. On July 25, the river discharge was estimated to be 250 c.f.s., and the maximum and minimum temperatures were 80° F. and 62° F. respectively. The method used to radioactivate larvae was essentially the same as that used in 1950. Radioactive larvae were released into the stream from July 6 to 26. A cubic-yard cage was placed over some of the larvae in the river. Adults were collected with sweep nets from July 9 to Aug. 23. A total of 234 collections were taken, the majority being within three miles of the experimental site on the river but some at distances as great as 30 miles. These collections were usually monitored on the day of collecting with a portable beta–gamma survey meter.

An estimated 700,000 larvae were treated in eight tests in 1950 (Table IV). Of this number approximately 500,000 were released into the Torch River, apparently in good condition.

The average radioactivity of larvae counted from each of these tests ranged from 3000 to 40,000 disintegretations per minute; 34% had an average of 22,000; 46%, 30,000; and 10%, 40,000.

Larvae placed in a treatment tub and examined at intervals showed a rapid and steady increase in their radioactivity (Fig. 8).

In treatment 5, approximately 12,000 larvae were placed in the solution at 6.00 p.m. on July 19, at which time the radioactivity of the solution was 0.143 μc. of P^{32} per ml. Two hours later, the radioactivity of the larvae averaged 77,400 disintegrations per minute; at 1.15 a.m., July 20, 194,000; at 6.15 a.m., 319,000; at 12.00 a.m., 370,000; and finally at 6.00 p.m., 380,000. Therefore, more than 97% of the total radioactivity of the larvae was obtained during the first 18 hr. of the 24 hr. treatment.

TABLE IV

RESULTS OF FIELD TREATMENTS* OF BLACK-FLY LARVAE** WITH RADIOACTIVE PHOSPHORUS, TORCH RIVER, SASKATCHEWAN, 1950

Treatment number	No. of larvae treated	Conc. of P^{32} (μc. per ml.)		Date of release. July	Radioactivity of released larvae (dis. per min.) $\times 10^{-3}$		Date of pupation, July	Date of emergence of adults, July
		Initial	Final		Average	Range		
1	50,000	0.200	0.019	15	41.1	130–10	—	—
2	40,000	0.021	0.009	17***	10.8	21– 4.2	15	—
3	45,000	0.021	0.009	18***	2.9	5.4–0.9	—	—
4	60,000	0.074	0.063	19	10.9	17.5–1.6	—	—
5	12,000	0.143	0.098	20	400	800–150	20	20
6	125,000	0.087	0.044	21	22.4	40–8	21	—
7	175,000	0.176	0.095	25	30	50–6	25	—
8	180,000	0.196	—	26***	—	—	26	—

* *All were 24-hr. treatments except for No. 2, which was a 40-hr. treatment.*
** *In tests 1 to 4, the species of larvae were Simulium venustum, S. luggeri, and S. vittatum in order of abundance. In the remainder, S. luggeri was the most abundant.*
*** *All of the larvae died during treatment.*

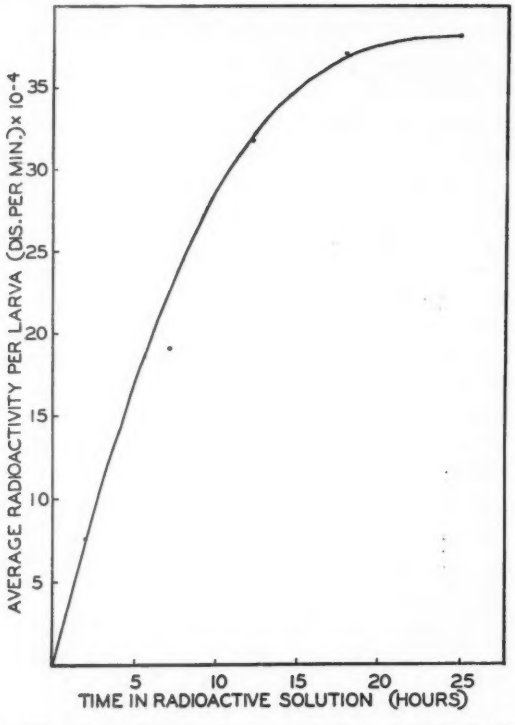

FIG. 8. Uptake of P^{32} by black-fly larvae kept in a solution containing 0.143 μc of P^{32} per ml.

The larvae in three of the eight tests (tests 2, 3, and 8) were dead at the end of their treatments. Larvae from the remainder of the treatments were in a healthy condition when released. The death of the larvae was attributed to lack of food in tests 2 and 3, and to too great a concentration of larvae in test 8. Supplementary feeding was not used in tests 1 to 3. Small quantities of yeast were provided in tests 4 to 7, and larger and perhaps excessive quantities were added in test 8.

Radioactive black-fly larvae and pupae were readily located in the stream with the portable beta–gamma survey meter. On July 18, three days after the release of the first group of treated larvae, radioactive larvae and pupae were found as far as 115 yd. downstream. The majority of these larvae were found in an area extending from 10 to 40 yd. from the point of release. On July 21, radioactive larvae and pupae were found as far as 252 yd. downstream from the point of release. The direct line of stream flow was determined by releasing several floating objects and tracing their course for about 150 yd. downstream. Larvae were not found outside this direct line of stream flow from the point of release.

The last group of radioactive larvae was released into the stream on July 26. Small numbers of radioactive larvae and pupae were last found in the stream on Aug. 4. None was found on Aug. 9, or on Aug. 22, when the last examination of the stream was made.

Radioactive pupae were first found in both the rearing tub and the stream on July 18. Pupae removed from the tub for test 5 on July 20 were placed in a jar in the stream, where they remained at stream temperature. Three adults emerged. On July 27 at 5.00 p.m. these black flies showed radioactivity values of 175,000, 495,000, and 506,000 disintegrations per minute. These were the only radioactive adults obtained in the field in 1950.

On July 26, bottles covered with radioactive pupae from tests 7 and 8 were placed in the stream and a netting-cage placed over them. On the same date, a rearing tub that had numerous radioactive pupae in it was also placed in the stream and covered with netting. No adults were recovered from either cage.

Radioactive adult black flies were not obtained out of captivity, despite daily sweepings for black flies around livestock and over vegetation along the river until Aug. 10, and despite the continuous use of light traps until Aug. 9. The last collection of black flies from livestock and from the river banks was made on Aug. 23. A total of more than 6000 black flies were collected.

In the second field test, made in 1951, a total of 300,000 black-fly larvae, predominantly *S. luggeri*, but also *S. venustum*, *S. vittatum*, *S. meridionale* Riley, and *S. rugglesi* N. & M., were made radioactive and released into the Battle River in west-central Saskatchewan (Table V).

It was planned to treat a much larger number of larvae than in the 1950 tests, but a scarcity of larvae prevented this. In these tests larvae in one treatment were lost, presumably as a result of high water temperature and overcrowding. In subsequent treatments, ice was added to the treatment solution during the hottest part of the day and no other losses occurred.

TABLE V

RESULTS OF THE TREATMENTS OF BLACK-FLY LARVAE* WITH RADIOACTIVE PHOSPHORUS, BATTLE RIVER, SASKATCHEWAN, 1951

Treatment number	No. of larvae treated	Conc. of P^{32} (μc. per ml.)		Date of release, July	Radioactivity of released larvae (dis. per min.) $\times 10^{-3}$	
		Initial	Final		Average	Range
1	10,000	0.2	0.05	6	61.6	178– 2.4
2	30,000	0.2	0.08	7	14.2	33– 4.2
3	30,000	0.2	0.06	8	48.9	90–12
4	35,000	0.2	0.05	10	66.3	170– 6
5	30,000	0.2	0.02	21	78.1	180–12
6	40,000	0.2	0.04	22	13.8	44– 3
7	100,000	0.2	0.02	23	18.9	120– 0.3
8	60,000	0.25	0.14	26	39.2	160– 8
9	40,000	0.14	0.05	27	11.9	32– 2.1

** Larvae in tests 1 to 4 were approximately 100% S. luggeri; in tests 5, 6, and 7, 87% S. luggeri, 8% vittatum, 2% venustum, 2% meridionale, and 1% rugglesi; in tests 8 and 9, 86% S. luggeri and 14% vittatum.*

Treated larvae were released into the stream from July 6 to 26. Radioactive larvae and pupae were found as far as 520 yd. downstream from the point of release.

On July 11 and 12 tagged adults emerged in the laboratory from radioactive pupae collected from the first lot of radioactive larvae released into the river on July 6. Commencing on July 16, a small number of tagged adults were taken from a cubic-yard cage placed over radioactive larvae and pupae in the river. A single radioactive adult, a female of *S. venustum*, was taken from a horse 100 yd. from the experimental site on July 25. Collections of adults were taken with sweep nets, commencing on July 9 and continuing almost daily until Aug. 23. In all, 234 collections were made. These contained an estimated 16,000 adults, most of which were females and of which only the above-mentioned one was radioactive.

Discussion

The foregoing experiments indicate that black-fly larvae can be tagged in large numbers with radiophosphorus with relative ease and with an economy of radioactive material. A large number of full-grown larvae of five species of black flies, *Simulium luggeri*, *S. vittatum*, *S. venustum*, *S. meridionale*, and *S. rugglesi*, were marked with sufficient P^{32} to be readily detected by a Geiger counter throughout the life of the insect. The marking was accomplished by transferring the larvae from their normal stream habitat to an aquarium or tub containing a small volume of the stream water to which P^{32} (as $P^{32}O_4$, approximately 0.2 μc. per ml.) had been added. After a 24 hr. exposure to the radioactive medium they were transferred to a nonradioactive medium or

into a stream for the completion of their development. Longer exposure did not increase the radioactivity of the larvae sufficiently to be worth-while, and shorter periods were not certain to provide effective tags. The tagging of a large number of black flies was feasible only in the late-instar larval stage, because pupae did not take up sufficient P^{32} to be detected with a Geiger counter, and the collection and activation of adults in large numbers was not practical.

The use of radioactive materials in experiments with black-fly larvae involved problems that would not be encountered in similar studies on terrestrial or pond insects. It was not feasible to treat an entire stream because, in addition to the danger of contamination, radiophosphorus is expensive and the supply limited. It was, therefore, necessary to limit the amount of water used to a few gallons. The aeration and circulation of small quantities of water was accomplished with air jets in the laboratory, and with stream-driven paddle wheels in the field. Small amounts of yeast added to the water eliminated the excessive mortality of larvae experienced in the first trials. When the proper environmental conditions of food and of water circulation and aeration were provided, black-fly larvae were successfully reared in small volumes of radioactive solution, numerous adults being highly radioactive. However, when larvae were similarly tagged in the field and returned to the stream, very few radioactive adults were obtained. More than 800,000 larvae were tagged and released into two streams, and it was proved that some of these established themselves in the streams, but only a single tagged adult was collected in the open. A few tagged adults were obtained from cages placed over larvae and pupae in the stream.

Although the reasons for the failure to collect tagged black flies in the field are not known, one or more of the following may have been factors: (*a*) radioactive larvae failed to establish themselves after release into the stream; (*b*) radioactivation prevented transformation to the adult or so weakened the flies that they failed to emerge from the stream; (*c*) disease and predation took such a heavy toll that only a very few of the radioactive larvae survived; or (*d*) the flies, on emergence, immediately dispersed over a wide area, reducing the chances of recovery by the collecting methods employed.

These factors cannot be adequately evaluated at present. The percentage of radioactive larvae that re-attached themselves in the stream was not determined. Colonies of radioactive larvae and pupae were, however, found without difficulty as far as 520 yd. downstream from the point of release.

The high rate of survival and of transformation of radioactive larvae to adults in the laboratory suggests that the radioactive phosphorus, in the quantities used, was not harmful to the insects. This view is also supported by the fact that the radioactivity established in individual specimens was well below the values found by Arnason *et al.* (1) to prevent transformation in *D. melanogaster*, and by Hassett and Jenkins (6) to be lethal to mosquitoes.

Mortality of nonradioactive larvae in the stream as a result of disease and predation was noted to be high, and was probably equally high among radio-

active specimens. The recovery in the 1950 collections of 21 adults of various predaceous stream-inhabiting insects, which could only have obtained radioactivity by predation on black-fly immature stages after their return to the stream, also suggests that natural enemies took a heavy toll of the tagged larvae. However, further studies of methods of releasing radioactive larvae appear to be required.

Theoretically, the chances of recovery of a tagged black fly are in indirect proportion to the square of the distance of flight from the point of release. If the species used in these tests made flights comparable to those recorded by Cameron (3) and Rempel and Arnason (9) for *S. arcticum*, another prairie species, then the methods used to recover tagged specimens were entirely inadequate. There was evidence that at least one of the species used in the tests had a relatively long flight range. Females of *S. luggeri* were collected as far as 26 miles from the nearest breeding places. On the other hand, feeding swarms of *S. venustum* were usually found within half a mile of the river from which they emerged, indicating that, providing suitable hosts were available, this species made only a short flight from the place of emergence; yet only one radioactive specimen was taken.

Most of the collecting was done by two to four assistants using hand nets around herds of livestock. This method of collecting practically eliminated the possibility of capturing any males, or even females if the species was not an habitual blood-feeder. Light traps were generally unsuccessful, presumably because of cool evenings, both in 1950 and 1951. A trap using carbon dioxide as an attractant was also unsuccessful. In general, from the poor results obtained, it appears that continuous collecting at a great many points radiating in all directions for several miles from the release site is necessary (perhaps taking into account wind direction and strength in the selection of sampling sites). It also appears necessary to develop a trap that attracts and retains most of the flies that appear in a relatively large area around it. Only with such traps will it be possible to make full use of any radioactive or other marking technique, unless a tremendous force of collectors is employed. Such traps should also be small in size and easily transportable.

Even though these preliminary studies have produced little information on the flight habits of black flies, they have shown that it is possible to tag a large number of black-fly larvae in a short time. They have also demonstrated the usefulness of radioactive tags for studying dispersion of larvae by providing definite proof that larvae are able to establish themselves after moving downstream considerable distances, and they have also shown their value as a means of identifying insect enemies of black flies.

It is believed that the basic procedures developed in these studies for the tagging of large populations of black-fly larvae will be successful in the study of flight range and dispersal habits of adults when a method has been developed for recapturing large numbers of them, and, with some minor modifications, that they would be equally effective in similar studies of other stream-inhabiting insects.

Acknowledgments

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PSEUDOCRUZIA (OXYUROIDEA : KATHLANIIDAE) A NEW GENUS OF NEMATODE FROM DOMESTIC SWINE IN INDIA[1]

By R. W. Wolfgang[2]

Abstract

Cruzia orientalis described from pigs in India is re-examined and after comparison with other species of *Cruzia*, all of which occur in American hosts, it is made the type of a new genus, *Pseudocruzia*.

In 1930, Maplestone (1) described from the caecum of domestic pigs in Calcutta, a new species of nematode which he called *Cruzia orientalis*. During the course of a revision of this genus, some of Maplestone's original specimens were sent to me by Dr. N. V. Bhaduri of the School of Tropical Medicine.

Although no males were present among the specimens sent (males were described by Maplestone), an examination of the head characters, which are common to both sexes, showed that they belonged to a different genus. The Indian specimens were compared with three species of *Cruzia* available to the author, namely: *C. tentaculata* (Rud., 1819) Trav., 1917; *C. testudinis* Harwood, 1932; and *C. cameroni* Wolfgang, 1951. The lips of these species have a definite shape and structure for each species but they are all alike in that they are simple, triangular, and bear no sort of cuticular inflation; moreover the stomal opening is small. The present genus differs in having interstomal papilliform processes as well as greatly inflated lips, a wide stomal opening, and a few minor characters which will be noted in the description. Owing to these differences a new genus is indicated for which the name *Pseudocruzia* is proposed, with *Pseudocruzia orientalis* (Maplestone, 1930) as type species. In view of variations between the description of the species by Maplestone and my own observations a brief redescription of the species seems desirable.

Fourteen females were available for examination. Medium-sized worms, dark in color (which may be due to long preservation). Length 10.82 to 17.03 mm. Lips (lateral length) 0.045 to 0.068 mm. The oesophagus is divided into four parts, beginning with the characteristic kathlanid pharynx which measures 0.24 to 0.30 mm. long. It is not much broader than the corpus and bears three longitudinal rows of 18 to 22 teeth, which project medially into the lumen instead of upwards as in *Cruzia*. They are about the same size and length throughout. The long corpus measures 2.55 to 2.62 mm. in the specimens examined and is followed by a small prebulbular swelling 0.10 to 0.12 mm. In *Cruzia* the prebulbular swelling is distinct from the corpus in the specimens examined, but in *Pseudocruzia* it appears to be a continuation of the corpus, no constriction being present to separate them.

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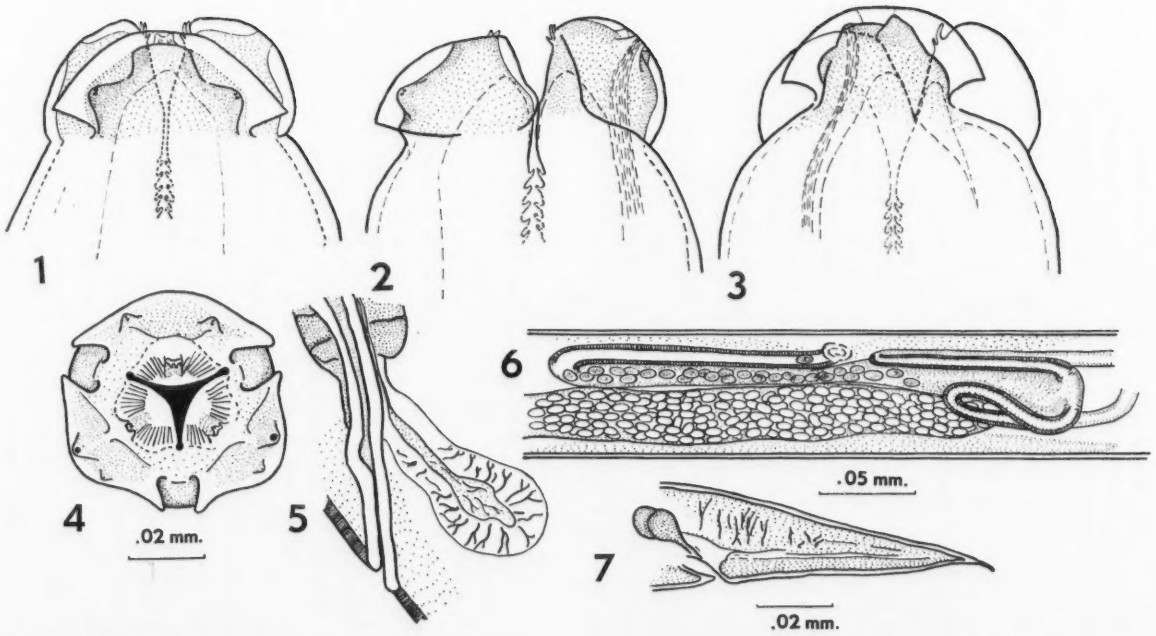
[2] *Research Assistant.*



Pseudocruzia orientalis

FIG. 1. Head end, dorsal aspect. FIG. 2. Head end, lateral aspect. FIG. 3. Head end, subventral aspect. FIG. 4. Head end, *en face* aspect. FIG. 5. Termination of female genitalia. FIG. 6. Female genital system. FIG. 7. Tail end of female.

The terminal oesophageal bulb is more or less pyriform and measures 0.27 to 0.32 mm. long. A diverticulum, similar to that found in *Cruzia*, measures between 1.0 to 2.0 mm. long. In all the specimens examined as well as in the figures given by Maplestone, the diverticulum twists about the oesophagus, not making a complete turn, but merely crossing the corpus at some point.

The intestine is simple except for a short expansion at the oesophago-intestinal junction. The tail is 0.69 to 0.73 mm. long (Fig. 7), broad at the anus, but tapering to a blunt point.

The ovaries are amphidelphic, the anterior one reaching to just below the oesophago-intestinal swelling, the posterior to about 1.5 mm. from the anus in gravid specimens. The uteri empty into the saclike vagina from the anterior (Fig. 6) by means of long, thin, muscular tubes. There is a long, muscular ovejector extending posteriorly, turning abruptly anteriorly, and terminating at the vulva about the linear middle of the body. The vulva (Fig. 5) is a simple orifice which is hardly noticeable in the present specimens owing to their dark color. Associated with the vulva there is a saclike extension which does not open into the lumen of the ovejector and which may be glandular or supportive in function.

The eggs are of the usual oxyuroid type, oval and embryonated when laid. In the gravid uterus they lie in masses but the presence in the ovejector of individual eggs widely separated from one another suggests that they are laid singly. They measure 0.12 by 0.05 to 0.06 mm. in the ovejector.

Discussion

The head of *Pseudocruzia* is distinct from the rest of the body, being separated by a constriction at the base of the lips. Figs. 1, 2, and 3 represent the head from the dorsal, lateral, and subventral aspects, respectively. The expansions appear to be pointed at the external angle when viewed from the lateral plane. This character can be seen even more clearly *en face* (Fig. 4), and is due to the expansion of the cuticle. The muscular portion of the lips is represented by the shaded area in the drawings. It will be noted in the lateral view that there are small interstomal papilliform processes which protrude out of the stomal opening. This character is at variance with *Cruzia*. Below the anterior processes is a pair of interstomal papillae.

Fig. 4 is an *en face* view showing the relative size of the lips and the arrangement of the papillae and amphids. The dorsal is the largest lip and bears a pair of double papillae, one on either side of the midline. The subventral lips are equal and bear on each an amphid laterally and a single papilla medially. The amphids are stalked and have large cores which run posteriorly toward the nerve ring. The enervation for the papillae is easily observed. There are two main trunks for the main lip and one each for the subventrals; these connect medially and send processes to the labial papillae. No anastomosis was noted before the main stems reached the nerve ring.

A recent paper by Ruiz (2) includes a key to the genus *Cruzia* together with a critical treatise on the characters of the genus. *Cruzia* is confined, with the

single exception of *Cruzia orientalis*, to the New World. It has a wide range of vertebrate hosts extending through amphibia, reptiles, edentates, and marsupials, but none so far removed as the artyodactyles.

The present study shows that morphologically the species in pigs of India constitutes a new genus. Its geographical and host distribution seem to support this separation. However, it is known that pigs in India are voracious eaters of snakes. Although Maplestone states that *C. orientalis* was taken in about 20% of the animals he examined, they were never present in large numbers (as is more usual in the case of *Cruzia*). Further investigation may establish a reptilian host for *Pseudocruzia* and show that it is a pseudoparasite of pigs.

Pseudocruzia gen. nov.

Generic diagnosis: Oxyuroidea : Kathlaniidae.

Medium-small worm, light in color, tapering at both ends. Head bearing three distinct lips which have a widely inflated cuticle. Wide stoma bearing double papilliform processes interlabially. Pharynx with three rows of teeth projecting at right angles into the lumen. An intestinal diverticulum present. Female with vulva near midbody, glands associated with vulva and tail. Eggs embryonated at laying. Male with equal spicules; papillae as in *Cruzia*. Other structures as in *Cruzia*.

Type: *Pseudocruzia orientalis* (Maplestone, 1930).

Host: *Suis suis*.

Location: Caecum.

Locality: Calcutta, India.

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THE CLYPEUS AND THE EPISTOMAL SUTURE IN HYMENOPTERA[1]

By E. Melville DuPorte and R. S. Bigelow

Abstract

The frontoclypeal suture in the Hymenoptera does not arch upwards into the frontal region but retains its primitive position at the horizontal level of the mouth, the clypeus remaining preoral in position. The genae and postgenae have continued or resumed their ventral growth, the inner margins of the genae have fused with the previously free lateral edges of the clypeus, and a pair of clypeogenal sutures, continuous with the frontogenal, have formed along the lines of fusion. The clypeogenal sutures form the vertical or oblique elements of the U-shaped epistomal suture, while the frontoclypeal suture forms its horizontal component. The anterior tentorial pits, within the bases of the frontogenal sutures, lie at the junction of the frontoclypeal with the clypeogenal sutures. These changes in the structure of the face have caused a ventral shifting of the mandibular articulations.

Introduction

The interpretation of the homologies of the head sclerites presents some of the most difficult problems in insect morphology. One of these problems is whether the sclerite called the clypeus in many of the higher insects is homologous with the clypeus of the generalized head such as found in the Orthopteroida and other primitive insects. We are concerned here with this problem as it relates to one order, the Hymenoptera. Our studies have been made chiefly on the Symphyta, the most primitive group of the order, because, as would be expected, this group indicates clearly the successive changes that have taken place in the evolution of the face. We have, however, examined a large number of species from other groups.

The conclusions presented here were reached independently by the two authors, by one from a study of the internal ridges of the face, by the other from his studies of the external sutures.

The Generalized Pterygote Clypeus

The relations of the clypeus in generalized Pterygota to the other structures of the face have been interpreted by DuPorte (1) as follows.

The clypeus (Figs. 1 and 7, *Clyp*) lies dorsal to the labrum and is separated from the remainder of the cranium by the transverse frontoclypeal suture (*fcs*), sometimes called the epistomal suture, which extends between the ventral ends of the two frontogenal sutures (*fgs*). These three sutures mark the positions of internal ridges or cuticular inflections. The frontogenal inflections (Fig. 6, *Fgi*) were formed, in the first place, by the infolding of the lateral edges of the facial plate in the Apterygota, and the anterior mandibular articulations of the Pterygota (Fig. 6, *aam*) were made with the edges of the

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face at the ventral ends of the inflections. The anterior tentorial arms (*At*) are attached to the inflections immediately dorsal to the mandibular articulations.

When the frontoclypeal inflection (Fig. 6, *Fci*) was formed at the level of the mouth, it joined the two frontogenal inflections at points adjacent to the origin of the tentorial arms, therefore externally the anterior tentorial pits (Fig. 1, *at*) lie at the junctions of the frontoclypeal suture with the frontogenal sutures.

When the lateral regions of the apterygote head grew ventrally to form the genae of the pterygote head the frontogenal inflections were marked externally by the frontogenal sutures which now separate the frons from the genae. This ventral growth ceased in the generalized insect at the level of the mouth, i.e., the anterior end of the definitive foregut. As a result, the proximal edge of the clypeus, the two pairs of mandibular articulations, and the ventral edges of the genae all lie in approximately the same horizontal plane as the mouth, and the clypeus is entirely preoral in position.

The lateral edges of the generalized clypeus are free, i.e., they are not united with any other cranial sclerites.

The Clypeus in the Hymenoptera

In most Hymenoptera, as in many other higher insects, the free lateral edges of the clypeus are reduced in extent or may be completely lost. The epistomal suture in these species is shaped more or less like an inverted U and its appearance suggests that the frontoclypeal suture has migrated dorsally into the frontal region. This, in effect, is the commonly accepted interpretation. Snodgrass (2), for example, states that this "suture is frequently arched upwards and this change in the position of the suture extends the clypeus into the facial region and reduces the area of the frons". Actually, since the greater the apparent arching of the suture the less is the extent of the free lateral edges of the clypeus, the sclerite gives the impression of having migrated bodily into the frontal region.

DuPorte (1) regards the preoral position of the clypeus as diagnostic and of more importance morphologically than the presence, absence, or position of the transverse suture which divides the face. He, like Snodgrass, believes that the frontoclypeal suture results from a purely functional inflection in a primitively single facial sclerite but holds that if it shifts dorsally it will lie across the frons, the sclerite ventral to it will no longer be entirely preoral, and, therefore, should not be regarded as the clypeus. Further, as an examination of Fig. 1 will show, if the frontoclypeal suture *(fcs)* shifts dorsally, it must move between the frontogenal sutures *(fgs,)* and the U-shaped suture thus formed will have these as its vertical components.

Snodgrass (3), in spite of his belief in the dorsal migration of the frontoclypeal suture, claims that the clypeus is still preoral and he has presented convincing evidence of this, drawn from his studies of the foregut musculature

and the position of the frontal ganglion. He does not commit himself as to whether the dorsal migration of the mouth follows that of the suture or *vice versa*.

We found it impossible wholly to accept Snodgrass's interpretation of the evolution of the clypeus because we could not conceive of any mechanism which would cause the position of the mouth to follow the wanderings of a purely functional fold in the outer wall of the head. Our study of the head structure in the Hymenoptera, however, has convinced us that, at least as far as this group is concerned, Snodgrass is correct in his concept of what constitutes the clypeus, but at fault in his explanation of its evolution and in his claim that the U-shaped epistomal suture, which bounds the clypeus dorsally and laterally, is merely the frontoclypeal suture in a changed position.

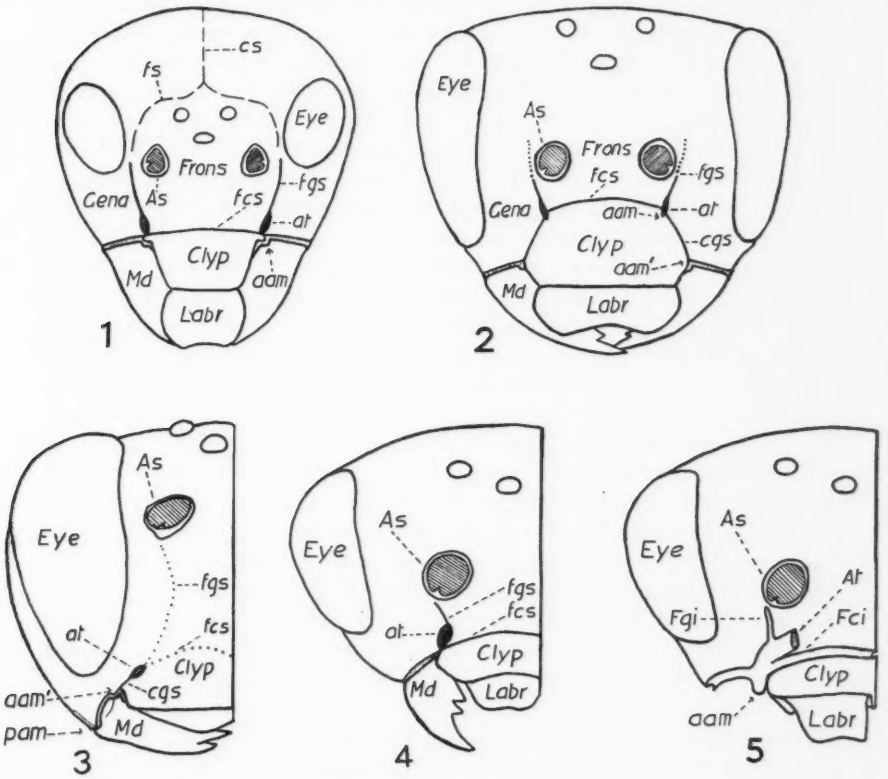
These studies have led us to conclude that the changed relationship of the clypeus to other facial structures has been brought about, not by the dorsal migration of the clypeus, but by a continuation of the ventral growth of the genal and postgenal regions of the head and the fusion of the mesal edges of the new genal regions with the previously free lateral edges of the clypeus. This fusion causes the formation of a pair of clypeogenal sutures (Fig. 2, *cgs*) lying between the genae and the lateral edges of the clypeus, and extending from the tentorial pits (*at*) to the anterior mandibular articulations (*aam'*). The clypeogenal sutures, therefore, are formed as the result of the continuation of the same process which resulted in the formation of the frontogenal sutures (*fgs*) and may be regarded as a ventral continuation of the latter beyond the tentorial pits.

It will be seen that since the frontoclypeal suture and the mouth retain their primitive positions the clypeus is still preoral.

If the schematic drawing of the generalized head (Fig. 1) is compared with that of the hymenopteron (Fig. 2), it will be seen that the frontoclypeal suture (*fcs*), the frontogenal sutures (*fgs*), and the tentorial pits (*at*) have the same relations to each other in both, but in the hymenopteron the ventral edge of the gena is no longer in the same plane as the frontoclypeal suture. It is evident that the frontoclypeal suture forms only the horizontal portion of the suture which borders the dorsal edge and much of the lateral edges of the clypeus; the portions containing the tentorial pits are parts of the frontogenal sutures, while the vertical or oblique portions are clypeogenal sutures. It is obviously incorrect to call this compound suture the frontoclypeal suture, but we suggest that the term "epistomal suture", though not accurately descriptive, can profitably be retained for it because of its somewhat less precise connotation.

With the descent of the genal and postgenal regions the posterior articulations of the mandibles are carried ventrally and, with the union of the clypeus and genae, the anterior articulations are forced to shift from their primitive positions (Figs. 1 and 2, *aam*) to a more ventral position (Fig. 2, *aam'*). In the Symphyta and probably most other species the two articulations are still at approximately the same horizontal level.

The individuality of the frontoclypeal, frontogenal, and clypeogenal inflections can be seen clearly in the larva of the sawfly *Macremphytus* (Fig. 8). Here the frontoclypeal (*Fci*) and frontogenal (*Fgi*) inflections are separated, but if the narrow gap between them is bridged, the relations of these inflections



ABBREVIATIONS IN FIGS. 1-21.

aam, primitive position of the anterior mandibular articulation.
aam', secondary position of the anterior mandibular articulation.
As, antennal socket or foramen.
At, anterior tentorial arm.
at, anterior tentorial pit or invagination.
Cgi, clypeogenal inflection.
cgs, clypeogenal suture.
Clyp, clypeus.
cs, coronal suture.
es, ecdysial suture.
Fci, frontoclypeal inflection.
fcs, frontoclypeal suture.
Fgi, frontogenal inflection.
fgs, frontogenal suture.
fs, frontal suture.
ic, clypeal inflection.
Labr, labrum.
Md, mandible.
pam, posterior articulation of the mandible.

FIG. 1. Facial view of the generalized pterygote head (schematic). FIG. 2. Head of a hymenopteron (schematic). FIG. 3. *Protichneumon grandis* (Br.). Ichneumonidae. Latero-frontal view of head to show mandibular articulations. FIG. 4. *Diprion (Gilpinia) hercyniae* (Htg.) Diprionidae. FIG. 5. *Diprion hercyniae*. Internal view of face.

to each other and to the tentorial arms (*At*) would be precisely the same as in the generalized head (Fig. 6). The level of the ventral edge of the cheek, however, has changed and, instead of being in the same plane as the proximal edge of the clypeus, it has almost reached the level of the distal edge. The broad inflection labelled *Cgi* is obviously a new feature of the face marking the union of the genal elongations with the lateral edges of the clypeus of which only a short portion now remains free. This new clypeogenal inflection is continuous with the frontogenal inflection, but there is a distinct groove between the two.

The individual inflections can be distinguished in the head of the honeybee (Fig. 9) also, but here, as in other adult Hymenoptera, the three are closely united to form the continuous epistomal inflection. Both of these figures (8 and 9) show how the distal end of the clypeogenal inflection (*aam'*) has replaced that of the frontogenal inflection (Figs. 6, 8, and 9, *aam*) in the formation of the condyle for the articulation of the mandible.

The antennae in the honeybee (Fig. 9, *As*) and in many other species lie close to the frontoclypeal suture. Since their primitive position is far dorsal to the suture (Fig. 1, *As*) this juxtaposition might be interpreted as evidence of the dorsal migration of the suture. Actually it is due to the ventral migration of the antennae. The position of the antennae is probably the most variable feature of the insectan head. In the very primitive and generalized head of the dermapteron *Anisolabis* (Fig. 7) for example, the antennae have migrated to a position similar to that in the honeybee. The same is true of the primitive sawfly *Pleroneura* (Fig. 14) in which the clypeus retains its primitive relations to the other parts of the head and there can be no question of a dorsal migration of the suture. Besides, in many of the more specialized Hymenoptera, in which the apparent dorsal migration is very pronounced, the antennae have retained their primitive position far dorsal to the frontogenal suture (Figs. 11 and 12).

Primitively the frontogenal suture extends dorsally lateral to the antenna and terminates between the antenna and the lower edge of the eye (Fig. 1). When the antennae migrated ventrally in *Anisolabis* (Fig. 7), there was no change in the suture, but only in one doubtful case among the sawflies examined did we find evidence of the suture continuing dorsally lateral to the antenna. In all other Hymenoptera examined the frontogenal suture terminates near the lateroventral edge of the antennal socket regardless of the distance between the antennae and the frontoclypeal suture. Frequently the inflection is fused with the inflected rim of the socket (Figs. 20 and 21). The length of the suture therefore varies with the distance of the antennae from the frontoclypeal suture (Cf. Figs. 15 and 17, *fgs*). It must be assumed, therefore, that a greater or lesser amount of the suture is lost dorsally and this loss is indicated in the larva of *Macremphytus* (Fig. 8) in which the dorsal portion of the inflection is a mere vestige which can be distinguished only

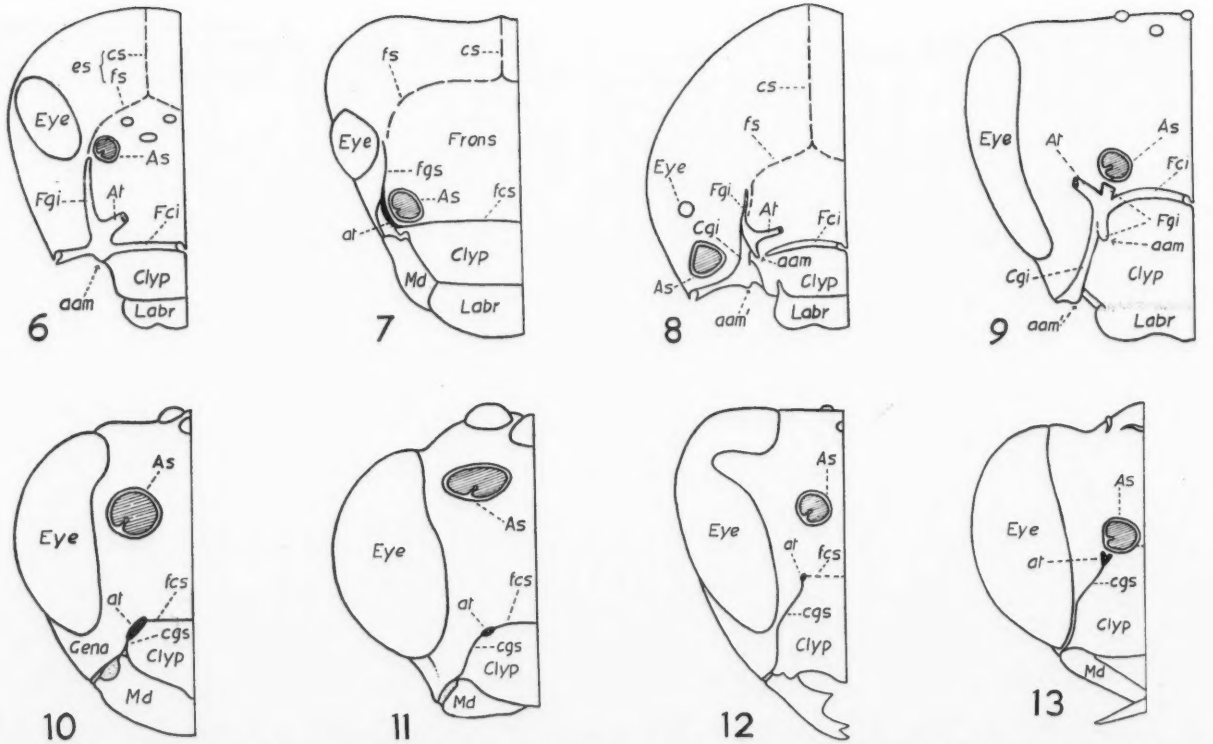


FIG. 6. Internal view of a generalized pterygote head (schematic). FIG. 7. *Anisolabis maritima*, Dermaptera. FIG. 8. *Macremphytus* sp., Tenthridinidae. Internal view of the face of larva. FIG. 9. *Apis mellifera* L., Apidae. Internal view. FIG. 10. *Thyreodon atricolor* (Oliv.), Ichneumonidae. FIG. 11. *Meteorus trachynotus* Vier., Braconidae. FIG. 12. *Polistes fuscatus* (Fab.), Vespidae. FIG. 13. *Bicyrtes ventralis* (Say), Sphecidae.

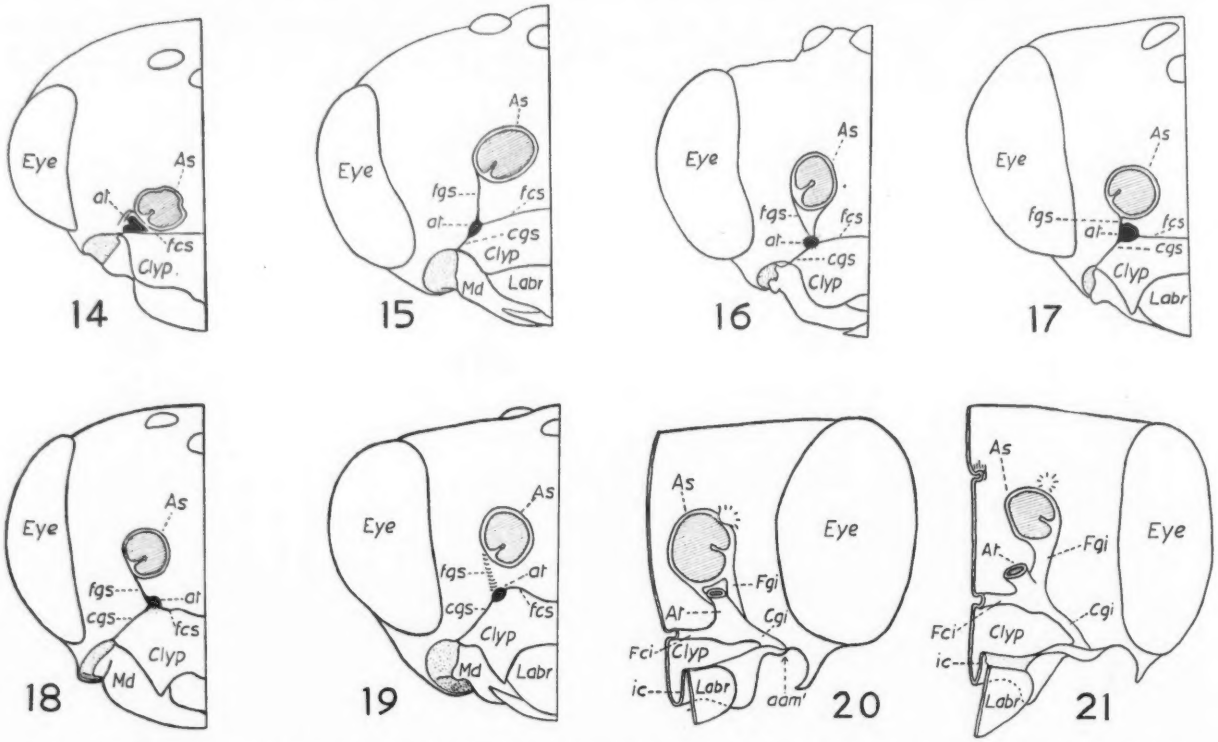


FIG. 14. *Pleroneura aldrichi* Ross, Xyelidae. FIG. 15. *Sphacophilus plumiger* (Klug), Argidae. FIG. 16. *Hemitaxonus albido-pictus* (Norton), Tenthridinidae. FIG. 17. *Ametastegia equiseti* (Fallen), Tenthridinidae. FIG. 18. *Ametastegia glabrata* (Fallen), Tenthridinidae. FIG. 19. *Macremphytus semicornis* (Say), Tenthridinidae. FIG. 20. *Hemitaxonus albido-pictus*, internal view. FIG. 21. *Ametastegia equiseti*, internal view.

with difficulty. In many of the more specialized Hymenoptera the suture dorsal to the tentorial pits is obsolescent (Fig. 3, *fgs*) or completely lost (Figs. 3, 11 and 12).

The frontogenal and frontoclypeal inflections reinforce that part of the cranium against which the mandible works. This strengthening device would be lost with the ventral shift in the position of the mandibular articulation, but the loss is compensated for by the development of the clypeogenal inflection and, in most species, by an infolding of the distal edge of the clypeus between the two articulations (Figs. 20 and 21, *ic*). This inflection carries the base of the labrum inwards and dorsally. In the honeybee (Fig. 9), where the mandibles are relatively weak, the ventral end of the clypeus is not thus inflected but there is a weak inflection of the very short free lateral edges.

The morphological gena is the region lying lateral to the frontogenal sutures (Fig. 1) and, primitively, ventral to the eye (1), but in most of the species examined the eyes are greatly enlarged and have grown ventrally into the genal regions. Indeed it appears that there is some correlation between the size of the eyes and the degree to which the genae have elongated, but we are not prepared at this stage to postulate any causal relationship between the two phenomena.

In many of the higher Hymenoptera the descent of the genae has continued until the entire visible lateral edges of the clypeus have been enclosed by them (Figs. 12 and 13) but in the Symphyta the distal half, at least, remains free. In fact some of the most primitive sawflies still retain the generalized facial structure. *Diprion hercyniae* (Figs. 4 and 5) is one such. Here the frontoclypeal suture, the mandibular articulation, and the ventral edge of the genae still lie approximately in the same plane. It is evident, on comparing the drawings of this head with those of the generalized head (Figs. 1 and 6) that, apart from differences in proportions, there is an almost exact correspondence between the two. In *Diprion* the anterior tentorial pit (Fig. 4, *at*) is relatively large which makes it possible to distinguish clearly that its location is in the frontogenal and not in the frontoclypeal suture.

It is possible to select among the Symphyta several series which show the progressive elongation of the genae and the parallel increase in length of the clypeogenal sutures. One of these series is illustrated in Figs. 14 to 19. *Pleroneura aldrichi* (Fig. 14) retains the primitive structure; the frontoclypeal suture and the ventral edge of the genae are in the same horizontal plane, the lateral edges of the clypeus are free, and there is no clypeogenal suture. *Sphacophilus plumiger* (Fig. 15) shows some elongation of the genae and has a short but distinct clypeogenal suture. The other members of the series have a progressively longer suture as the genae elongate more and more. In the more highly specialized species the genae and clypeus are closely fused externally, with a genuine suture between them, but in some of the more primitive species, while they are united internally to form the inflection, externally the groove is still open and fusion of the lips of the inflection is not complete.

In conclusion it should be pointed out that if the theory that the clypeus in Hymenoptera is formed as a result of the dorsal arching of the frontoclypeal suture is accepted, several other assumptions must be made. It must be assumed that as the suture shifts dorsally there is a corresponding shift in the position of the mouth. Since the extent of the free portion of the clypeus becomes less and less as the apparent arching increases, it must be assumed that the arching is accompanied by a coincident and proportional reduction in the distal portion of the clypeus. When the apparent arching is pronounced it must be assumed that the tentorial arms have shifted their position within the head to a considerable extent and this must result in several changes in the internal structures of the head.

The interpretation given here, on the other hand, involves no greater assumption than the continuation of a process which is known to have taken place in the evolution of the pterygote from the apterygote head, namely, the ventral growth of the lateral regions of the head and the fusion of these regions with the lateral edges of the primitive facial plate. The ventral shifting of the mandibular articulations follows as a natural consequence. That the genae and postgenae do elongate ventrally in the Hymenoptera can be clearly seen by reference to the drawing of the head of the ichneumonid *Protichneumon grandis* (Fig. 3). In this insect, as in other ichneumonids, the posterior articulation of the mandible (*pam*) has been carried so far ventrally that the dicondylic hinge of the mandible is now vertical instead of horizontal. This condition admits of only one interpretation, that the postgena and lateral portion of the gena have grown ventrally carrying the posterior articulation of the mandible to a vertical instead of a horizontal position as related to the anterior articulation.

Summary

The primitive clypeus is preoral and is bounded dorsally by the frontoclypeal suture which extends across the face between the bases of the frontogenal sutures. The tentorial pits lie in the frontogenal sutures at the junction of the latter with the frontoclypeal suture.

The frontoclypeal suture in the Hymenoptera retains its primitive horizontal position at the level of the mouth. It does not arch upwards into the frontal region, therefore, the clypeus remains preoral in position.

The genae and postgenae have elongated ventrally and the mesal edges of the genae have fused with the lateral edges of the clypeus. A pair of clypeogenal sutures is formed along the lines of fusion and extends from the tentorial arms to the anterior mandibular articulations.

The epistomal suture consists of the horizontal frontoclypeal suture, the bases of the frontogenal sutures containing the tentorial pits and the two vertical or oblique clypeogenal sutures.

With the descent of the genae and postgenae and the fusion of the genae and clypeus both mandibular articulations have shifted ventrally.

The position of the antennae varies and is of no significance in interpreting the clypeus.

The frontogenal sutures are reduced dorsally and extend only to the rim of the antennal socket. In many species they are lost beyond the tentorial pits.

In most species the eyes are greatly enlarged and extend ventrally into the genal regions.

The processes described are merely a continuation of those which resulted in the evolution of the pterygote from the apterygote head.

Acknowledgments

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ÉTUDE SUR LES ACIDES AMINÉS LIBRES DE L'HÉMOLYMPHE DES INSECTES PAR LA MÉTHODE DE CHROMATOGRAPHIE SUR PAPIER FILTRE[1]

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Abstract

A qualitative and semiquantitative analysis of the free amino acids in the hemolymph of nine insect species was carried out with the method of paper partition chromatography. Details concerning the above method as applied to insect blood analysis are given. A discussion of the results obtained with those published in the literature is included. From 16 to 24 free amino compounds are regularly identified in insect blood, many of them in high concentration. There are qualitative and quantitative differences in amino acids between different insect species. Quantitative analyses of the free amino nitrogen of insect hemolymph are presented at the end of the paper. These quantitative determinations were carried out by means of the Van Slyke gasometric apparatus using the nitrous acid reaction.

Introduction

Les acides aminés occupent une place d'intérêt primordial dans l'étude du chimisme de l'être vivant. Une vingtaine de ces substances existent sous forme de métabolites libres aux niveaux cellulaire et humoral, mais se rencontrent surtout comme constituants fondamentaux des protéines plastiques et enzymatiques. Leur métabolisme complexe est encore lié à celui d'autres substances essentielles non protéiniques, telles que les purines, la créatine, les hormones adrénaline, thyroxine, etc. Les acides aminés constituent un matériel d'oxydation énergétique, directement ou par le détour des glucides, et une source de lipides et d'ammoniac par désamination. Ils sont enfin des produits nécessaires dans le mécanisme de la détoxication et de l'excrétion.

Une des principales caractéristiques biochimiques de la classe des Insectes est la forte teneur de leur milieu intérieur en acides aminés libres (2, 6, 7, 12, 15, 16, 18, 19, 20, 21, 22, 23, 25, 27, 29, 30, 32, 33, 34). Cette particularité, chez des organismes qui se prêtent très bien aux expériences en laboratoire, présente un grand intérêt pour l'étude du métabolisme de ces substances. La concentration en acides aminés de l'hémolymphe des insectes est de 10 à 40 fois plus élevée que celle du sang de tout autre groupe animal (24). Alors que, chez les mammifères, cette concentration ne dépasse guère 0.5 gm. par litre de plasma, chez les insectes, elle atteint couramment de 5 à 20 gm. par litre d'hémolymphe.

L'identification des composés aminés libres n'a été que très récemment entreprise en détail, et sauf pour un ou deux acides aminés, il n'existe pas de données quantitatives sur ces substances. Ranga-Rao et Sreenivasaya (31) ont trouvé 2.5% de tyrosine sous forme libre dans la partie hydrosoluble de

[1]*Manuscrit reçu le 25 septembre, 1952.*

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Lakshadia mysorensis. Florkin et Duchateau-Bosson (25) établissent les teneurs en tyrosine et en histidine de l'hémolymphe de *Dytiscus marginalis* à 117–168 mgm./100 ml. et 31–35 mgm./100 ml. respectivement. Ces données quantitatives s'avèrent très incomplètes lorsque l'on sait que de 15 à 20 acides aminés ou plus peuvent être identifiés dans le sang des insectes, et que la teneur en azote aminé du plasma varie au cours du cycle ontologique de chaque espèce (12, 16, 20, 23, 25, 27).

Les seules données que nous connaissons sur la variation qualitative des acides aminés libres selon les états de développement ont été publiées par Pratt (30), qui a établi la distribution de ces composés dans le sang de la larve et de l'adulte de la mouche domestique.

La présente étude comporte une identification des acides aminés libres de l'hémolymphe de neuf espèces d'insectes par la méthode de chromatographie sur papier filtre, une analyse semiquantitative de ces composés par la même méthode, et finalement, une détermination quantitative de l'azote aminé dans l'hémolymphe de cinq espèces d'insectes par la méthode gazométrique de Van Slyke utilisant l'acide nitreux.

Méthodes expérimentales

1. *Espèces étudiées et modes d'élevage*

Les cinq premières espèces qui apparaissent dans le Tableau I ont été élevées en laboratoire sur les milieux nutritifs décrits dans ce tableau. Les quatre autres espèces ont été prélevées durant l'été sur leurs hôtes naturels et la saignée effectuée immédiatement. Les plantes servant de nourriture à ces quatre dernières espèces sont présentées dans le même tableau.

2. *Prélèvement des échantillons d'hémolymphe*

Afin de prévenir la coagulation de l'hémolymphe, les insectes sont immergés durant trois à cinq minutes dans un bain d'eau à la température de 62 à 65° C. Le sang d'au moins 10 individus est obtenu en perçant délicatement le tégument au moyen d'une fine pointe de verre, ou en coupant le bout d'une patte ou d'une fausse patte selon les facilités qu'offre l'espèce à l'essai. Le sang, aspiré dans une micropipette graduée (micropipette Neale-Forbes) est mêlé et employé tel quel, ou dilué avec de l'eau distillée, ou dilué avec de l'alcool à 95% (4) lorsqu'il est nécessaire de précipiter les protéines et divers autres composés. Dans ce dernier cas, l'échantillon d'hémolymphe est dilué à 10 fois son volume avec l'alcool et le précipité est séparé par centrifugation. Cette extraction est répétée trois fois sur le précipité, les fractions alcooliques sont évaporées complètement à pression réduite à la température du laboratoire. Au moment de l'analyse, le résidu sec est repris dans l'eau distillée au volume initial de l'échantillon.

3. *Analyse qualitative des acides aminés*

L'emploi de la microméthode d'analyse chromatographique sur papier filtre, introduite par Consden, Gordon, et Martin (11), permet la résolution et

TABLEAU I

INSECTES ÉTUDIÉS ET RÉGIMES ALIMENTAIRES

Espèces	Régimes alimentaires
Galleria mellonella (L.) (Teigne des ruches)	Pablum imprégné d'un mélange de miel, de glycérine et d'eau (2 : 2 : 1)
Tenebrio molitor L. (Ténébrion meunier)	Son de blé, pommes séchées, eau
Drosophila melanogaster L. (Drosophile)	Eau 100, agar-agar 2, crème de blé 3, "Nipagin M" 1, levure 6, mélange X 3 *Mélange X:* sucrose 25, peptone 10, extrait de levure 5, KH_2PO_4 1.
Blatta orientalis L. (Blatte orientale)	Biscuits "Purina", eau
Epilachna varivestis Muls. (Coccinelle mexicaine des haricots)	Feuilles de *Phaseolus vulgaris* L. (haricot)
Phlegethontius quinquemaculatus (Haw.) (Sphinx de la tomate)	Feuilles de *Lycopersicon esculentum* Mill. (tomate)
Archips cerasivorana (Fitch) (Tordeuse du cerisier)	Feuilles de *Prunus cerasus* L. (cerisier)
Malacosoma americana (F.) (Livrée d'Amérique)	Feuilles de *Prunus cerasus* L. (cerisier)
Leptinotarsa decemlineata (Say) (Doryphore de la pomme de terre)	Feuilles de *Solanum tuberosum* L. (pomme de terre)

l'identification rapides de tous les acides aminés présents dans un mélange complexe. Cette méthode, qui a été utilisée dans le présent travail, est très efficace pour l'étude qualitative et semiquantitative des acides aminés dans l'hémolymphe des insectes.

Le développement des chromatogrammes bidimensionels est effectué par ascension capillaire (36) avec les mélanges phénol-eau et collidine–lutidine–eau comme solvants. Ces mélanges sont préparés de la façon décrite dans un travail précédent (2). A la suite du développement au phénol, les papiers sont séchés pendant plusieurs heures dans un courant d'air à la température de la pièce. Cette façon d'opérer prévient la destruction partielle des acides aminés par l'action du phénol (10, 26). Après le développement à la "collidine", les papiers sont séchés de nouveau. Les chromatogrammes sont révélés par traitement avec une solution de ninhydrine à 0.1% dans le *n*-butanol et par chauffage dans une étuve maintenue entre 70 et 80° C. pendant 10 à 15 min.

L'identification des acides aminés déduite de la distribution des taches colorées apparaissant sur le chromatogramme a généralement été confirmée par l'addition d'acides aminés purs sur les chromatogrammes expérimentaux. L'hydrolyse (14) de l'échantillon étudié a de plus précisé l'identification des

amides glutamine et asparagine et éliminé toute équivoque due à la présence possible de peptides. L'identification des acides aminés sulfurés (i.e. la méthionine) a été vérifiée par l'emploi des tests à l'iodoplatinate de potassium (37) ou à l'oxydation au peroxyde d'hydrogène (14). Pour ce dernier test, des volumes égaux de peroxyde d'hydrogène à 30%, de molybdate d'ammonium à 0.02%, et de l'échantillon de sang sont mêlés. Le mélange est chauffé à environ 50° C. jusqu'à évaporation de la majeure partie de la solution. Le résidu est alors repris dans l'eau distillée et porté sur le papier filtre. La présence de la taurine dans le sang de certains insectes a été vérifiée par le test au carbonate de cuivre récemment décrit par Crumpler et Dent (13). Finalement, certaines expériences sur le développement des chromatogrammes en atmosphère acide ou alcaline, ou sur l'emploi de solvants directement acidulés ou alcalinisés, ont aidé à l'identification des acides aminés basiques lysine, arginine, et histidine.

4. *Préparation des solutions d'acides aminés purs*

Les solutions d'acides aminés purs employées pour vérifier les identifications établies sur les échantillons de sang ont été préparées dans l'alcool isopropylique à 10% (8). Des mélanges d'acides aminés en concentration optimum dans ce solvant permettent la préparation rapide de plusieurs séries de chromatogrammes standards à deux dimensions.

5. *Détermination de l'azote aminé*

La méthode Van Slyke à l'acide nitreux (35) a été utilisée pour les dosages de l'azote aminé libre du sang de cinq espèces d'insectes.

Résultats des analyses chromatographiques

Les données récemment acquises sur la nature des acides aminés en solution dans le sang des insectes ont été obtenues surtout par l'emploi de la méthode de chromatographie sur papier filtre (1, 2, 4, 15, 18, 19, 22, 29, 30, 32). Avec cette méthode, nous avons étudié la distribution des acides aminés du sang des insectes suivants: quatre lépidoptères, *Galleria mellonella*, *Phlegethontius quinquemaculatus*, *Malacosoma americana*, et *Archips cerasivorana;* trois coléoptères, *Tenebrio molitor*, *Leptinotarsa decemlineata*, et *Epilachna varivestis*; un diptère, *Drosophila melanogaster* et un orthoptère, *Blatta orientalis*. Toutes les analyses de sang ont été effectuées sur le dernier stade larvaire des espèces, à l'exception de *B. orientalis* étudié au stade adulte. En raison de la difficulté d'obtenir un volume de sang quelque peu abondant des larves de la drosophile, il est possible que la liste des acides aminés chez cette espèce demeure incomplète.

1. *Résultats qualitatifs*

Les hémolymphes furent soumises à des analyses détaillées, effectuées sur des échantillons minimes de sang entier (0.02 à 10 µl.) employés tels quels ou en dilution aqueuse, et sur des échantillons traités à l'alcool tel que décrit précédemment, en quantité allant de un jusqu'à 300 µl. dans certains cas.

TABLEAU II

COMPOSÉS AMINÉS IDENTIFIÉS DANS L'HÉMOLYMPHE D'INSECTE*

Composés aminés	Lépidoptères				Diptère	Orthoptère	Coléoptères		
	G. mellonella	*P. quinquemaculatus*	*M. americana*	*A. cerasivorana*	*D. melanogaster*	*B. orientalis*	*L. decemlineata*	*T. molitor*	*E. varivestis***
α-Alanine	0.05	0.25	0.5	0.5	0.25	0.5	0.5	0.2	+
Acide aspartique	0.25	0.5	5	5	0.25	1	2	0.2	+
Glutamine	0.05	0.25	0.5	0.5	0.25	0.5	0.25	0.2	+
Acide glutamique	0.05	0.12	1	0.5	0.25	0.12	0.25	0.2	+
Glycine	0.3	0.12	0.5	1	0.35	0.25	0.5	0.8	+
Leucine et/ou *isoleucine*	0.25	0.5	2	8	0.35	1	1.5	0.4	+
Lysine	2.5	0.5	2	3	1.5	12	1	10	+
Méthionine	1	2	20	10	1	3	3	2	+
Proline	0.5	1.5	5	5	0.75	2	1	0.6	+
Sérine	0.2	0.25	0.5	0.5	0.35	1	0.5	0.6	+
Thréonine	0.8	1.5	4	8	2	1	3	1	+
Tyrosine	1.5	0.5	5	5	0.75	2	3	1	+
Valine	0.2	0.5	1	3	0.5	1	0.5	0.4	+
Acide α-amino-*n*-butyrique	30	20							
Arginine	6	3	1	5	0.75	1	1	0.8	
Asparagine	4.5				1.5		1		+
β-Alanine	2	2	15	8		5		12	+
Cystine et/ou cystéine	50	50	75	25			50		
Histidine	5	13	15	20		7	30	2	
Hydroxyproline					5				
Taurine						1.5		4	
Tryptophane	55						10	15	+
"Under aspartic"	2			8	1	1		1.5	
"Under glutamic"	0.2			1	0.75	1	0.25	0.6	
"Over threonine"	1	0.5		3		100			
Volume maximum de sang chromatographié sur un chromatogramme	150	300	150	100	10	100	100	100	100

* *Les nombres indiquent, en microlitres, le volume minimum d'hémolymphe correspondant à l'apparition du composé aminé sur le chromatogramme.*
** *Volumes minima non déterminés pour cette espèce.*

Les résultats du Tableau II indiquent que 13 ou 14 composés aminés se retrouvent dans toutes les espèces analysées, soit: l'alpha-alanine, l'acide aspartique, la glutamine, l'acide glutamique, la glycine, la leucine (et/ou isoleucine), la lysine, la méthionine*, la proline, la sérine, la thréonine, la tyrosine, et la valine. Les noms de ces composés sont en caractères italiques dans le Tableau II. A l'exception de la tyrosine, ces composés ont déja été identifiés au moyen de la même méthode d'analyse dans le plasma humain (9). Ce groupe de 13 ou 14 composés apparaît presque invariablement lorsque de 2 à 5 µl. d'hémolymphe sont chromatographiés en deux dimensions.

D'autres acides aminés ou dérivés, tels l'acide alpha-amino-*n*-butyrique, l'arginine, l'asparagine, la beta-alanine, la cystine (révélée sous forme d'acide cystéique après oxydation par le peroxyde d'hydrogène), l'histidine, l'hydroxyproline, la taurine, et le tryptophane, ont été reconnus chez une ou plusieurs des espèces analysées. Les résultats négatifs présentés dans le Tableau II signifient soit l'absence, soit la présence en concentration extrêmement faible des substances concernées. Quelques composés non identifiés réagissant au test de la ninhydrine ont aussi été rencontrés quelques fois au cours des analyses. Parmi ces substances, un composé qui semble correspondre à l'"under glutamic" de Awapara *et al.* (5) a souvent été observé, présentant une tache d'intensité souvent très considérable. Enfin, quelques essais préliminaires avec un mélange de butanol, d'acide acétique, et d'eau pour la seconde dimension chromatographique, ont permis de mettre en évidence la présence de l'acide gamma-amino-*n*-butyrique dans le sang de quelques espèces d'insectes, dont *Blatta orientalis* et *Tenebrio molitor*. Ce mélange a déjà été utilisé par un des auteurs pour l'identification de l'acide gamma-amino-*n*-butyrique dans le miellat excrété par une espèce de puceron (3). Ce mélange nous a aussi permis de reconnaître la phénylalanine chez *Galleria mellonella*.

La majorité des auteurs ne mentionnent pas tous les composés aminés retrouvés chez toutes les espèces analysées dans le présent travail. Employant des méthodes chimiques pour la résolution des acides aminés du sang de *Oryctes nasicornis* et *Melolontha vulgaris*, Ussing (34) ne décèle pas l'alanine, la glycine, la thréonine, la méthionine, la sérine, la proline, et les acides aspartique et glutamique. Raper et Shaw (32) et Finlayson et Hamer (22) ne donnent pas la méthionine, la thréonine, l'acide glutamique, et son amide la glutamine pour la nymphe d'*Aeschna cyanea* et la larve de *Calliphora erythrocephala*. Raper et Shaw (32) mentionnent cependant la présence d'une substance non identifiée: "- - - relatively large amounts of a substance, possibly a peptide, which runs at the same speed as alanine in phenol but somewhat slower in collidine". Cette description pourrait fort bien correspondre à la glutamine. Enfin, l'acide aspartique n'apparaît pas dans la liste présentée pour la nymphe d'*A. cyanea*. Levenbook (29) ne mentionne pas la glutamine ni la sérine comme composants du sang de *Gastrophilus intestinalis*. Drilhon

** La méthionine se retrouve surtout sous forme de "méthionine-sulfoxyde" sur le chromatogramme développé. D'après Block (8), cette oxydation se produirait pendant le développement au phénol lorsque la température ambiante dépasse 25° C.*

(15) présente la distribution des acides aminés libres dans l'hémolymphe de 13 espèces d'insectes. La glutamine, la méthionine, et la thréonine ne sont indiquées en aucun cas. Les acides aspartique et glutamique, la lysine, et la proline ne sont que rarement identifiés. Pratt (30) dans son analyse de sept espèces d'insectes ne décèle pas l'acide aspartique chez *Periplaneta americana*, non plus que la lysine et la thréonine chez cet insecte et chez la larve de *Musca domestica*. Auclair (1) et Pratt (30) ne mentionnent pas la thréonine chez *Oncopeltus fasciatus*.

Les résultats positifs obtenus par les auteurs sur la distribution des composés aminés moins courants sont comparables à ceux présentés dans le Tableau II. L'amide asparagine identifié dans le sang de plusieurs insectes (Tableau II), avait déjà été reconnu comme constituant de l'hémolymphe (1, 30, 33, 34), de même que l'acide alpha-amino-*n*-butyrique (1, 30), les acides aminés basiques arginine et histidine (1, 15, 22, 30, 34), la cystine (15, 30), l'hydroxyproline (15, 30, 34), le tryptophane (1, 15, 30), et la phénylalanine (22, 29, 30). Pratt (30) est apparemment le seul auteur à mentionner la beta-alanine et la taurine comme composants de l'hémolymphe d'insecte. Dans le présent travail, ces composés ont tous été retrouvés (Tableau II).

De cette revue sommaire sur les principales données actuellement disponibles concernant la distribution des acides aminés dans l'hémolymphe des insectes, il serait probablement prématuré de tirer des conclusions générales quant à des différences qualitatives entre les divers groupes d'insectes. Ces données fragmentaires, souvent incomplètes, portent sur trop peu d'espèces dont quelques-unes furent étudiées à des stades différents de développement. Cependant, de telles différences entre les divers groupes systématiques ou écologiques restent à prévoir.

Par contre, on peut déjà noter certaines différences dans la distribution des acides aminés de l'hémolymphe d'une même espèce prise à des stades successifs de développement. Pratt (30) identifie la cystine, la lysine, la thréonine, et la beta-alanine chez l'adulte de *Musca domestica* sans avoir décelé leur présence dans le sang de la larve et l'arginine rencontrée chez celle-ci ne se retrouve pas chez l'adulte. Nous avons effectué une analyse comparée du sang de *Galleria mellonella* à différentes périodes de son cycle ontologique. Les résultats obtenus seront présentés dans un travail ultérieur. La variation dans les distributions observées est relativement peu marquée et ici encore, les résultats négatifs obtenus pour certains stades n'indiquent peut-être qu'une concentration extrêmement faible des substances recherchées. Tel peut être le cas de la taurine et de l'hydroxyproline, présentes en assez grande abondance chez la chrysalide de *G. mellonella*, mais non décelables chez la larve (Tableau II). Les 20 autres composés aminés identifiés dans le sang de cette espèce se retrouvent à tous les stades analysés.

L'interprétation physiologique de la présence de ces quelque 24 composés aminés dans le sang des insectes reste généralement hypothétique. Elle se fonde largement sur des analogies avec les quelques données expérimentales que nous possédons chez les mammifères. La présence des acides aminés non

reconnus comme faisant partie des molécules protéiniques est brièvement commentée dans l'intéressant travail de Pratt (30). Nous référons aussi aux études de Drilhon, Busnel, et Vago (19) et Drilhon et Busnel (17, 18) qui présentent les variations dans la distribution des acides aminés plasmatiques du ver à soie relativement à la race ou à certains états pathologiques.

2. *Résultats semiquantitatifs*

Bien que les analyses chromatographiques résumées dans le Tableau II n'aient pas été faites dans un but strictement quantitatif, on obtient une bonne approximation de la concentration des acides aminés en comparant les volumes minima d'hémolymphe du Tableau II avec les quantités minima publiées par Auclair et Dubreuil (2). Cependant le présent travail fut terminé avant la mise au point de la microméthode quantitative mentionnée ci-dessus (2) de sorte que la comparaison ne peut avoir qu'une signification semiquantitative.

En comparant les volumes minima du Tableau II avec les quantités minima déjà publiées (2), la concentration approximative de chaque acide aminé par 100 µl. d'hémolymphe d'insecte, varie selon l'ordre quantitatif suivant:

Pour l'alanine, de 12 µgm. (*M. americana* et quelques autres espèces) à 120 µgm. (*G. mellonella*).

L'acide aspartique, de 4 µgm. (*A. cerasivorana* et *M. americana*) à 100 µgm. (*T. molitor*).

La glutamine, de 80 µgm. (*M. americana* et quelques autres espèces) à 800 µgm. (*G. mellonella*).

L'acide glutamique, de 10 µgm. (*M. americana*) à 200 µgm. (*G. mellonella*).

La glycine, de 5 µgm. (*A. cerasivorana*) à 42 µgm. (*P. quinquemaculatus*).

La leucine et/ou isoleucine, de 3 µgm. (*A. cerasivorana*) à 100 µgm. (*G. mellonella*).

La lysine, de 12.5 µgm. (*B. orientalis*) à 300 µgm. (*P. quinquemaculatus*).

La proline, de 30 µgm. (*M. americana* et *A. cerasivorana*) à 300 µgm. (*G. mellonella*).

La sérine, de 8 µgm. (*B. orientalis*) à 40 µgm. (*G. mellonella*).

La thréonine, de 2.5 µgm. (*A. cerasivorana*) à 25 µgm. (*G. mellonella*).

La tyrosine, de 20 µgm. (*M. americana* et *A. cerasivorana*) à 200 µgm. (*P. quinquemaculatus*).

La valine, de 5 µgm. (*A. cerasivorana*) à 75 µgm. (*G. mellonella*).

A l'exception de la méthionine, cette première énumération comprend les composés aminés présents chez toutes les espèces étudiées. La détermination semiquantitative de la méthionine est difficile à cause de la superposition possible de l'acide gamma-amino-*n*-butyrique sur les chromatogrammes développés à la "collidine".

La concentration approximative des composés aminés identifiés chez quelques espèces seulement varie selon l'ordre quantitatif suivant:

Pour l'acide alpha-amino-*n*-butyrique, de 0.4 μgm. (*G. mellonella*) à 0.6 μgm. (*P. quinquemaculatus*).

L'arginine, de 67 μgm. (*G. mellonella*) à 533 μgm. (*D. melanogaster*).

L'asparagine, de 18 μgm. (*G. mellonella*) à 80 μgm. (*L. decemlineata*).

La beta-alanine, de 1.5 μgm. (*M. americana*) à 11 μgm. (*P. quinquemaculatus* et *G. mellonella*).

La cystine et/ou cystéine, de 0.27 μgm. (*M. americana*) à 0.8 μgm. (*A. cerasivorana*).

L'histidine, de 25 μgm. (*L. decemlineata*) à 375 μgm. (*T. molitor*).

L'hydroxyproline, 20 μgm. chez *D. melanogaster*.

La taurine, de 5 μgm. (*T. molitor*) à 13 μgm. (*B. orientalis*).

Le tryptophane, de 3.6 μgm. (*G. mellonella*) à 20 μgm. (*L. decemlineata*).

Les concentrations approximatives des composés aminés peuvent être groupées en une série décroissante comme suit:

Glutamine	80	à	800	μgm./100μl.	
Arginine	67	à	533	"	"
Histidine	25	à	375	"	"
Proline	30	à	300	"	"
Lysine	12.5	à	300	"	"
Tyrosine	20	à	200	"	"
Acide glutamique	10	à	200	"	"
alpha-Alanine	12	à	120	"	"
Acide aspartique	4	à	100	"	"
Leucines	3	à	100	"	"
Asparagine	18	à	80	"	"
Valine	5	à	75	"	"
Sérine	8	à	40	"	"
Glycine	5	à	42	"	"
Hydroxyproline			20	"	"
Thréonine	2.5	à	25	"	"
Tryptophane	3.6	à	20	"	"
Taurine	5	à	13	"	"
beta-Alanine	1.5	à	11	"	"
Cystine	0.27	à	0.8	"	"
Acide alpha-amino-*n*-butyrique	0.4	à	0.6	"	"

La glutamine est le composé dont la teneur est la plus élevée dans le sang des insectes, variant de 80 à 800 μgm./100 μl., soit 0.08 à 0.8%. Cet amide est un métabolite important chez les plantes et les animaux, où sa fonction principale serait le maintien de la réserve du groupe NH_2.

Les acides aminés basiques arginine, histidine, et lysine se maintiennent à une concentration élevée variant de 12.5 μgm. pour la lysine à 533 μgm. pour l'arginine. Ces trois acides aminés jouent un rôle primordial dans la nutrition et le métabolisme intermédiaire des mammifères. Ils sont essentiels dans la diète de quelques insectes tels que *Tribolium confusum*, *Aedes aegypti*, et *Drosophila melanogaster* (28).

La cystine et/ou cystéine (identifiées sous forme d'acide cystéique) et l'acide alpha-amino-*n*-butyrique n'existent qu'à l'état de traces dans le sang des insectes. La concentration de l'acide cystéique varie de 0.27 à 0.8 µgm./100 µl., tandis que la teneur en acide alpha-amino-*n*-butyrique est de l'ordre de 0.2 à 0.4 µgm./100 µl., ou 0.0002 à 0.0004%. La détection de ces composés en concentration extrêmement faible illustre bien la sensibilité de la méthode chromatographique dans l'ultramicroanalyse.

En général, la teneur de l'hémolymphe en acides aminés reconnus essentiels dans les études de nutrition est assez élevée relativement à celle des autres composés aminés. Ces études de nutrition ont démontré que les acides aminés essentiels remplissent un rôle important au cours de la métamorphose et de la reproduction chez les insectes.

Détermination de l'azote aminé par la méthode gazométrique de Van Slyke

La méthode Van Slyke utilisant la réaction à l'acide nitreux (35) a été employée pour les dosages de l'azote aminé libre de l'hémolymphe de cinq espèces d'insectes. Le sang fut prélevé tel que mentionné précédemment et dilué avec un volume égal d'eau distillée. Des échantillons de 150 à 300 µl. du mélange ont été analysés. Les résultats sont résumés dans le Tableau III.

TABLEAU III

AZOTE AMINÉ SOLUBLE DE L'HÉMOLYMPHE DE CINQ ESPÈCES D'INSECTES

Espèce	Stade larvaire	Nombre d'analyses	Azote aminé mgm./ml. (moyenne)
Malacosoma americana (F.) (Livrée d'Amérique)	Avant-dernier	4	3.57
Malacosoma americana (F.) (Livrée d'Amérique)	Dernier	4	3.03
Archips cerasivorana (Fitch) (Tordeuse du cerisier)	Dernier	2	3.48
Phlegethontius quinquemaculatus (Haw.) (Sphinx de la tomate)	Dernier	3	2.30
Galleria mellonella (L.) (Teigne des ruches)	Dernier	3	3.28
Leptinotarsa decemlineata (Say) (Doryphore de la pomme de terre)	Dernier	3	3.04

Les résultats présentés au Tableau III indiquent chez *M. americana* une diminution de la teneur totale en azote aminé libre de l'avant-dernier au dernier stade larvaire. Ces résultats diffèrent de ceux que l'on trouve ordinairement dans la littérature (20, 25, 27).

Chez quatre des espèces étudiées, trois lépidoptères, et un coléoptère, la concentration de l'azote aminé libre de l'hémolymphe ne varie que de 3.03 à 3.48 mgm./ml., bien que ces espèces aient des régimes alimentaires différents (Tableau I). La concentration en azote aminé atteint un niveau relativement bas de 2.30 mgm./ml. chez *P. quinquemaculatus*. Cette valeur faible peut être attribuée à l'influence du régime alimentaire, et aussi au métabolisme particulier de cette espèce.

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SOME EFFECTS OF TEMPERATURE ON THE EMBRYONIC DEVELOPMENT OF THE SALMON (*SALMO SALAR*)[1]

By F. R. Hayes[2], D. Pelluet[3], and Eville Gorham[4]

Abstract

This is a study of the effects of temperature on morphogenesis, and is an attempt to determine whether the order of appearance of anatomical features can be altered within the limits of survival. The time in days (usually from closure of the blastopore) for features to appear was noted, and from this the rates were calculated, being the reciprocals of days $\times 10^3$. Rate plotted against temperature gives in general a straight line whose slope may be calculated. Some slopes were: hatching, 4.6; digestive system, 6.4; skeleton, 6.5; nervous function, 8.2; external pigment, 9.6; blood vessels, 10.0; fins, 10.3; eye pigment, 12.5. Thus hatching would be expected to appear precociously at low temperatures, and eye pigment at high temperatures.

This work constitutes a study of some effects of temperature on the development of the salmon embryo. It is a further investigation along lines begun by Hayes and Pelluet (5), and represents an attempt to determine whether the order of appearance of morphological features during development may be altered by the use of different developmental temperatures within the limits for survival.

Methods

Salmon eggs were obtained at intervals from the Dominion Government Hatchery at Bedford, N.S. They were established in a small hatchery in the Zoology Laboratory at Dalhousie University. From the university hatchery batches of eggs were transferred for experimental purposes to controlled temperature chambers.

In earlier experiments the chambers were as described by Hayes and Pelluet (5), two methods being used to rear the eggs. In one method 10 eggs were placed in a 2-oz. bottle with a thin layer of tap water and an atmosphere of oxygen over them. Ten of these bottles were placed in each chamber. This method was only partially successful, since there was a high mortality just previous to and continuing after, hatching. Up to this point development appeared normal except that in the coldest chambers the embryos sometimes died at an earlier stage. The second procedure was to place a layer of eggs in a glass dish 24 cm. in diameter, filled with tap water plus 10% Halifax sea water to a depth of 6 cm. The water was then aerated by means of an aquarium pump or by compressed air. With this treatment eggs were hatched and reared to the fry stage, but utilization of all the chambers was not

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possible since the air jet interfered with the temperature difference between them. To obtain sufficient data to determine the most suitable graphic plot for the temperature relation, it was necessary to use the first method, and to restrict the experiments to the earlier features of development. The second method served to determine something of the extent and nature of the dissociations throughout the course of development.

For later experiments a refrigerated laboratory was available. Eggs were placed on standard hatchery trays in 8-gal. aquaria and the water was frequently changed. Air was bubbled into the tanks and a constant temperature was maintained in each by a heater whose setting was adjusted by a variable transformer. Heat was applied continuously and the fluctuation in water temperature was well within one degree. The system was protected by a thermostat designed to turn off all heaters if the room began to warm up. Great care was taken to avoid thermal and mechanical shocks, overcrowding, and oxygen deficiency, and it was anticipated that only a low mortality would occur. In this we were dissappointed because, for reasons not yet clear, no batch of eggs was ever brought through from early stages to completion of yolk sac absorption. We have been unable to find a satisfactory substitute for running water. It is obvious that the account which follows would have been more fully documented if single batches of eggs could have been kept alive for long intervals.

Eggs can be transferred to experimental tanks either in the first two or three days after fertilization, or after the blastopore has closed. Attempts to handle eggs between these dates results in heavy or total mortality.

Examination was carried out with a binocular microscope at intervals of from one to six days, depending on the temperature at which the experiment was being conducted. From 2-10 eggs were looked at in each examination, the capsule being removed in most cases. The yolk sac was left intact at the beginning of the examination but was usually removed later.

Eggs were taken from the University or Bedford hatcheries and placed under different controlled temperatures in nine main experiments, of which the following description of one will serve as an example:— Jan. 28, 1946. Eggs brought from Bedford hatchery and established immediately under 11 temperatures from 1.3° C. to 14.5° C. Degree of development approximately that described by Pelluet (12) as stage 6, vitelline vein prominent with unpigmented blood flowing through it. The eggs were reared in 2-oz. bottles under oxygen, and the number of days to reach various points in development, as marked by the appearance of certain morphological features, was recorded for each temperature. Some of the results are shown in Fig. 1. While errors in observing the number of days for a structure to appear may in some cases be quite considerable, those in determining the order of events are not as great, since it was generally found that embryos at the same temperature showed the same order of events even when the number of days varied.

Temperature Relations

The effects of temperature sometimes appear initially as a rate (heart beat, oxygen consumption, etc.) and sometimes as a time (days to hatch and other data in this paper). One kind of measurement is the reciprocal of the other. Thus if, as often happens, a rate curve is approximately a straight line, the corresponding time curve will be a portion of a rectangular hyperbola. We find it more convenient to deal with straight lines than curved lines, and have therefore, by taking their reciprocals, converted the attainment times in this paper into rates. The slope of a rate curve is a measure of the effect of temperature on reaction velocity and permits comparisons to be made between different processes. Table I gives the method used for calculating slopes.

TABLE I

DATA ON THE APPEARANCE OF BILE IN LUMEN OF GUT AT VARIOUS TEMPERATURES*

	1 Temp., degrees C., x	2 Days for bile to appear, y	3 Rate, being 1000 divided by days, v	4 Temp. multiplied by rate. Col. 1 × Col. 3, xv	5 Temp. or Col. 1 squared, x^2
	4.8	40	25	120	23
	6.2	25	40	248	38
	8.3	19	53	440	69
	9.8	13	77	755	96
	11.3	11	91	1028	128
	12.2	10	100	1220	149
	13.1	8	125	1638	172
	13.9	8	125	1738	193
	14.5	8	125	1813	210
Sum	94.1		761	9000	1078
Mean	10.5 M_x		85 M_v	1000 M_{xv}	119 $M(x^2)$
Mean squared	109 $(M_x)^2$				

**Illustrated in Fig. 1 on line marked (2). The slope of the rate curve, k_1, is calculated by the method of least squares, being given by the equation*

$$k_1 = \frac{M_{xv} - M_x \cdot M_v}{M(x^2) - (M_x)^2}$$

$$= \frac{1000 - 10.5 \times 85}{119 - 109} = 10.8$$

where the values of the symbols are as indicated in the above table.

NOTE: *Col. 2 is not used in the calculation of slope, being included here merely to exhibit the original data. In this example all the temperatures and rates were used, but for other rates shown in Fig. 1, where lines are inflected to horizontal at higher temperatures, the last points were omitted from the slope calculations.*

The relationship between time and temperature, described in the foregoing paragraph, is known as the rule of thermal summation. The rule states that the time, y, to reach any stage of development, multiplied by the temperature, x, is a constant, i.e. yx equals k. Often another constant has to be added or subtracted from the temperatures before the rule of thermal summation holds. This relation is the same as that which Krogh (8) found applicable to development in a variety of animals (fishes, amphibia, insects, and echinoderms), where the plot of rate versus temperature yielded a straight line.

Fig. 1 gives graphic pictures of the relation between differentiation and temperature. They have been made from the Jan. 28, 1946, experiment described above. Both time and rate plots have been constructed, and from the latter it is clear that the linear rate–temperature relation forms an adequate expression for the data over the greater part of the range.

It is interesting to note the points at which there is an actual divergence from the straight line treatment. That at which the curve for half-vascularization of yolk breaks away is near 9° C. (No. 5 on graph). Other lines break at

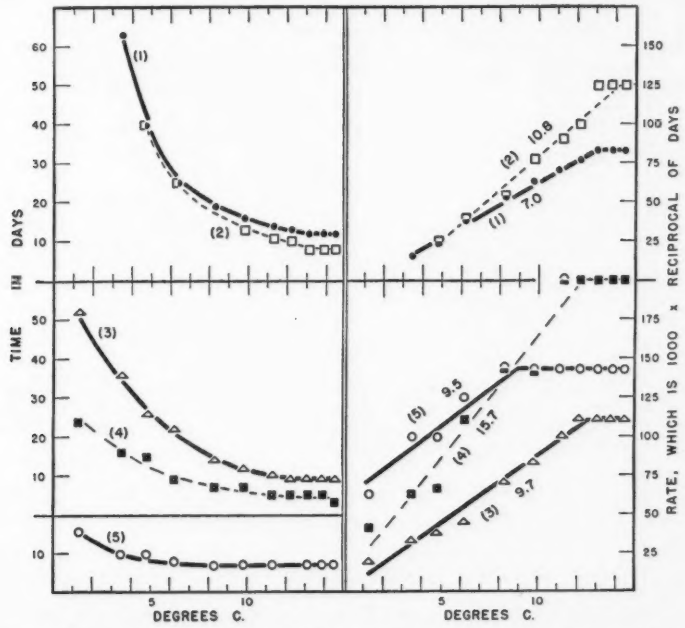

FIG. 1. Effect of temperature on the development of five morphological features. The experiment was started at approximately the time of blastopore closure. Left side of figure is days to attain the feature. The right side is a plot of reciprocals of the days $\times 10^3$, which are rates. Numbers beside the lines are slopes of the rate curves. Note that at high temperatures the accelerating effect of heat no longer operates. The point where the rate curve breaks varies from one process to another. The morphological features are identified by numbers as follows: 1. Establishment of tail circulation. 2. Appearance of bile in lumen of gut. 3. Completion of yolk sac circulation. 4. Development of pigment in the eye. 5. Yolk sac more than half vascularized.

11–13° C. Some rate lines actually cross one another, e.g., 1–2 and 4–5. The slopes of the rate curves, which have been entered on Fig. 1, give quantitative expression to the effect of temperature on the processes described. Measurements on embryonic weight gain or yolk loss, such as those of Hayes and Pelluet (5), can also be fitted quite well by rate curves. Observations covered a 10-day period sometime after hatching, during which a linear relation held, as shown in Fig. 2.

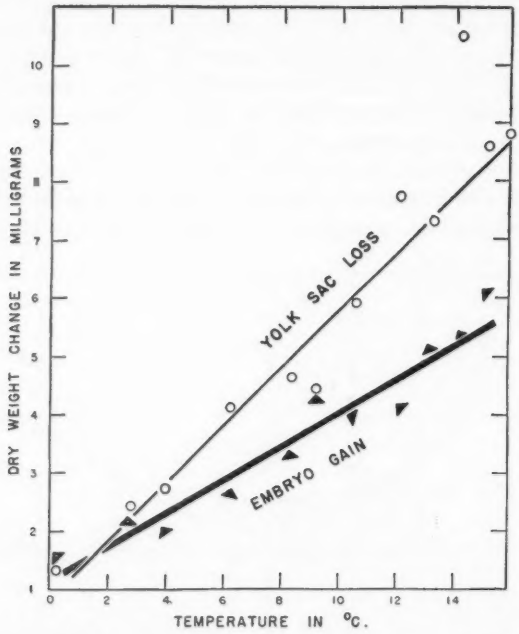

FIG. 2. Yolk loss or embryo gain in dry weight during a 10-day period of the post-hatching interval. Eggs were reared in flat-bottomed dishes containing ½ in. of water, and kept in temperature chambers. In this case rate was the effect directly observed and the relation is seen to be linear. Data of Hayes and Pelluet (1945).

In Table II are included the slope constants of various rate curves. These are averages calculated from several series of observations.

Dissociation

Some indication of a dislocation of differentiation processes in the salmon has been given by previous studies in this laboratory. Hayes (4) suggested that various factors, one of which is temperature, may cause the gland cells producing the hatching enzyme to secrete earlier than usual, and Pelluet (12) found the hatching period to occur rather variably with regard to designated developmental stages. An actual "uncoupling of embryonic processes" was also described by Hayes and Pelluet (5) in a study of temperature effects on

growth. However, their experiments dealt only with nonappearance of fat in the gut mesentery and failure of the gut to turn, at the warm and cold extremes respectively.

An inspection of Fig. 1 and of Table II reveals clearly that differences in the order of appearance of structures (as distinct from a complete inhibition) may be obtained by allowing development to proceed at different temperatures within the limits for survival. Thus at high temperatures the bile became noticeable in the widened lumen of the gut before the completion of the yolk circulation and establishment of blood vessels in the tail. At lower temperatures it slowed down relative to the other two processes, which overtook it. Thus at 3.5° C. it had not appeared when the period of mortality occurred in this experiment shortly before hatching, although both the other features had been observed. A similar reversal in the appearance of eye pigment and the time at which the yolk sac became half-vascularized may also be observed in Fig. 1. As has been seen, the rate (reciprocal of time for feature to appear) varies for the most part directly with the temperature, and a comparison

TABLE II

EFFECT OF TEMPERATURE ON THE RATES OF DEVELOPMENTAL PROCESSES. FIGURES GIVEN ARE THE SLOPES OF RATE CURVES. RATE IS DEFINED AS 1000 TIMES THE RECIPROCAL OF THE TIME TO ATTAIN EACH MORPHOLOGICAL FEATURE. COMPOSITE VALUES FROM ALL EXPERIMENTS

I. Hatching		4.6
II. Differentiation of the digestive system, average		6.4
Appearance of bile in lumen of gut	9.3	
Formation of stomach	7.0	
Appearance of folds or coils in intestine	6.4	
Fat seen in mesentery of gut or in spleen	3.0	
III. Development of the skeleton		6.5
Cartilage seen in dorsal and anal fins	7.5	
Rays seen in caudal, dorsal, anal, and pectoral fins	5.5	
IV. Development of nervous function as measured by mobility		8.2
Beating of pectoral fins	10.0	
Jaws seen to be moving	6.3	
V. Development of external pigment		9.6
Black chromatophores on head	10.4	
Black chromatophores on various parts of body	5.6	
Red and yellow chromatophores at several places	12.9	
VI. Development of blood vessels		10.0
Yolk half vascularized	9.5	
Yolk circulation complete	8.4	
Development of capillary system in tail	11.7	
VII. Appearance of external structures		10.3
Caudal fin	11.5	
Dorsal fin	12.7	
Pelvic fins	6.7	
VIII. Black pigment showing in eyes (Development of visual function?)		12.5

of the slope values of the rate curves for these processes which are presented in Table II, shows that the value for eye pigment exceeds the value for vascularization of the yolk. Since these processes are occurring at approximately the same time, the high value for eye pigment indicates that it is more affected by temperature change than is vascularization of the yolk, being faster at the high and slower at the low temperatures.

We might conclude in a general way from Table II, that low temperatures favor precocious hatching and differentiation of the digestive system and skeleton. On the other hand, high temperatures promote the completion of a functional circulatory system and external features such as fins and pigment. The maximal high temperature effect observed was on eye pigment. Whether this indicates a development of visual function or not is a question. Other evidences of nervous function, such as beating of fins, were intermediate in their response to temperature.

Regarding hatching, it is well-known in hatcheries that a very prolonged cold winter prevents normal hatching although the egg capsules give way and the embryos die while emerging. Normal hatching proceeds by the tail breaking through the capsule first. In cold experiments many embryos were found hatching head first or yolk sac first, with a consequent pinching of the yolk sac and body. They usually died, although in a few instances embryos were observed to hatch head first and survive, and to be developing normally when observed several days later. None was observed to hatch yolk sac first and live.

A rather striking effect of temperature is illustrated in the following example. Pelluet described stage 15 as consisting of (*a*) the appearance of pyloric caeca, (*b*) the appearance of black pigment patches on the sides, and (*c*) a heavy deposition of fat in the gut. In the present study, it was found that at 11.4° C., the fat and caeca appeared long before the pigment patches, which were observed to be forming at about the time the yolk was completely absorbed. In a later experiment, at 14.1° C. (started from a later stage), the embryos failed to show any sign of patches before the yolk was absorbed and they died of starvation. In contrast to this, at 6.6° C. fat appeared first, the pigment patches appeared while the yolk sac was still prominent, and the caeca in the gut did not form until several days later.

Abnormalities are produced by extremes of temperature at many stages in fish development (Stockard (14)) and the effect varies with the stage. When eggs were developed at 11.5° C. (starting at approximately stage 6 of Pelluet) the hatched embryos showed a marked flexure of the neck and tail over the yolk sac, so that normal swimming movements were impossible. A similar result was obtained by subjecting slightly more developed eggs to a temperature of 15.5° C. In both these experiments there was a high mortality during and following hatching, but more severe at the higher temperature. However, when eggs of the second lot were kept at 11.5° C. they survived without a high mortality at hatching. At first a slight flexure was noticed, but later it disappeared. It appears as though at 11.5° C. the critical period

is around stage 6, and the effect is still manifested to a slight degree at a somewhat later period. If this later stage is subjected to an even higher temperature however, the full effect of the abnormality may be restored. Finally, eggs were placed at 14.1° C. from approximately stage 11 of Pelluet. None of these exhibited any bending of the body after hatching, and developed without great loss to the fry stage; the critical period had been passed. Bəttle (1), in work on the teleost *Enchelyopus*, also finds that the degree of abnormality varies with the severity of the treatment. It therefore seems to be an effect on the rate relations of processes rather than an "all or none" phenomenon. Some degree of regulation was evidenced in the second lot at 11.5° C., since they returned to normal later on. Extreme powers of recovery from temperature-produced abnormalities are recorded for an amphibian (Coghill (3)).

Discussion

We may conclude from the above experiments that dislocations in the order of differentiation are possible with a change in the temperature at which development takes place, and that certain of these may take place within the normal limits for survival, although in the present study there has not been a survival to the fry stage in every instance.

It is probable that, in the embryo at least, these limits are governed at many stages by the amount of dissociation which can be tolerated between processes fundamental to the well-being of the embryo. Other factors, such as oxygen diffusion (Hayes *et al.* (6)) may of course be limiting at some points of development.

Heat death in adult organisms has been held by some workers to be due to protein coagulation or to some effects on the fats or lipids of the cell. Certain objections have been raised to such an hypothesis by Cameron (2), who believes that in highly organized animals the cause of death may be attributed to some change damaging the co-ordinating mechanism. This might in turn be due to a different susceptibility to temperature change in the rates of the various metabolic processes.

The fact that the thermal limits of embryos are generally narrower than those of adults, and may vary from stage to stage (Battle (1)), suggests that effects on the proteins or lipids are not the main factors involved here, although they may appear secondarily. It is more likely that, owing to a differential action on the rates of certain fundamental processes of metabolism, a degree of unbalance is reached at these limits which cannot be tolerated by the embryo. Wood (16) finds that in the trout the relations of the fundamental processes of respiration and growth in weight are unchanged over the temperature range normal to the fish, but that above and below this range respiration increases relative to growth and a smaller embryo results.

This unbalance of rates at the limits of the range may cause the production of monsters before the true lethal limit is reached. Stockard (14), who worked a great deal in this field, believes that unless they are produced by hereditary

factors, deformities are all caused by temporary inhibition of the rate of development (a "developmental arrest") which deranges the relative gradients of certain processes. The deformity varies with the time of inhibition because of the differences in relative metabolic gradients at the different stages of development.

The dislocations discussed above are also similar to certain dissociations of the major developmental processes of growth, determination, and differentiation reviewed by Needham (11), although his examples have generally been the result of unnatural conditions. The work of Twitty (15) is also relevant. He found by grafting experiments that the polarity of ectodermal ciliary beat in amphibians was normally determined at the closure of the neural folds, but with low temperatures determination took place at a much earlier stage in development.

Additional evidence that there is a differential effect by temperature may be derived from the work of Ljubitzky and Svetlov (10), who observed that succeeding stages in the formation of the pectoral fin in the trout responded differently to temperature. Similar results were subsequently obtained by Ljubitzky (9), for embryo formation in the trout egg.

That the end product of differentiation may be changed with a difference of developmental temperature has been shown by Schmidt (13) and Hubbs (7) in fishes. The latter worker found that in a year class which had developed in a cooler year than usual, the average number of vertebrae, scales along the lateral line, and branched anal rays was increased. This may be construed as supporting the general thesis that temperature affects certain processes differently during development.

The reasons that phenomena similar to those here described have not been apparent in many studies of temperature relations of embryos may be many. Simultaneous features selected as stage markers may be of such fundamental importance to the embryo that any dislocation would result in death, or at least in the production of monstrosities. In this case the temperature coefficients would necessarily be the same. Or features may have been selected which must follow one another, so that their order could not be reversed. Cases of this type are the use of successive stages of cleavage, blastula formation, and gastrulation, and of somite number and embryo lengths. Another is the identification of stages based chiefly on increase of specialization in one particular feature such as the external form of the limb bud. In these cases the temperature coefficients need not correspond. From the results of the present study we may conclude that stages of development drawn up for one temperature may not represent a true relation in all features when the developmental temperature is altered.

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THE CHEMOTACTIC RESPONSES THAT DETERMINE HOST SPECIFICITY IN AN OLIGOPHAGOUS INSECT (*PLUTELLA MACULIPENNIS* (CURT.) LEPIDOPTERA)[1]

By A. J. Thorsteinson[2]

Abstract

The mustard oil glucosides, sinigrin, sinalbin, and glucocheirolin and the mustard oil, allyl isothiocyanate, as well as the enzyme myrosin, were prepared from the seeds of cruciferous plants. *Pieris brassicae* (L.) and *Plutella maculipennis* (Curt.) were induced to feed on leaves of plant species which they normally refuse by painting the leaves with a solution of sinigrin or sinalbin but would not feed on such leaves treated with allyl mustard oil. Feeding responses of *P. maculipennis* larvae were tested on agar gels containing the powdered, dehydrated leaves of various plants alone and in combinations with glucosides, mustard oil, and myrosin. The amount of feeding was estimated by counts of frass pellets produced by the larvae during the test period. It was shown that sinigrin, sinalbin, and glucocheirolin are feeding stimulants for *P. maculipennis* larvae. Since the larvae feed readily on at least 40 plant species which are reported to contain mustard oil glucosides it was concluded that the mustard oil glucosides are specific feeding stimulants for this insect. The threshold concentrations for *P. maculipennis* of gustatory perception of sinigrin under these conditions are of the order of 2 p.p.m. for sinigrin and about 20 p.p.m. for sinalbin. Optimum feeding responses were obtained only when the glucoside is offered in a medium containing other nutrients in the form of powdered, dehydrated leaves or artificial mixtures. In some experiments the addition of allyl mustard oils slightly increased feeding on media containing sinigrin. The addition of viable myrosin to diets containing sinigrin decreased the feeding responses. Since heat-killed myrosin has no repellent effect, it appears that the action of myrosin is due to the depletion by hydrolysis of the quantity of sinigrin in the medium. Apparently the fission products of the hydrolysis including mustard oil are less attractive than the parent glucosides. Since hydrolysis of glucoside will release only minute amounts of mustard oil in the short space of time intervening between biting and swallowing it is unlikely that the gustatory receptors will be exposed to appreciable concentrations of mustard oil as compared with glucoside. On the other hand it is possible that infinitesimal amounts of mustard oil vapor emanating from leaves may stimulate the olfactory sense which is characteristically extremely sensitive in insects. While hunger induces sustained feeding in the absence of mustard oil provided a gustatory stimulant is present, such an olfactory stimulus might conceivably initiate feeding more promptly. This would account for observations in some experiments that larvae produced more frass when feeding on media containing a little mustard oil as well as sinigrin. The power of sinigrin to induce *P. maculipennis* to feed on nutrient media makes possible the development of an artificial medium for studies of the nutrition of this phytophagous insect.

Introduction

Host specificity in an oligophagous insect is manifest in two ways. The gravid female shows discrimination in its choice of a plant on which to oviposit. The larvae will accept as food the leaves of only a limited and frequently related number of plant species. Both phenomena are believed to consist

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of a chain of responses to optical, chemotactic, and tactile stimuli (32). The object of this study is to investigate the responses to chemical stimuli in an oligophagous insect in relation to the choice of host plants. The emphasis is on the feeding responses of the larvae to specific chemical constituents of their food plants. Studies of artificial food media and the olfactory responses of larvae and adults will be described elsewhere.

Chemical stimuli may be olfactory or gustatory. Probably olfactory stimuli largely determine oviposition sites. In the choice of food by larvae, on the other hand, while odor is probably the important initial stimulus as suggested by Dethier (7) it appears that gustatory phenomena finally determine whether sustained feeding will occur.

In both olfactory and gustatory responses, repellent as well as attractant substances play a part in the determination of host specificity. Indeed, Wardle (30) suggests that the term "host avoidance" may often be more accurate than "host selection". This may be true for polyphagous insects. However, in oligophagous insects the operation of both positive and negative chemotactic stimuli may determine host selection. For example, *Leptinotarsa decemlineata* (Say) feeds on *Solanum tuberosum* L. but is unable to survive on *S. demissum* Linn. (20). Both plants apparently contain a constituent that induces feeding but the latter contains in addition an alkaloidal glycoside, demissin, which inhibits feeding owing either to repellent or toxic action (3, 4, 20). The meaning of the term "oligophagy" as used in this paper is that suggested by Dethier (5) viz, host selection determined primarily by positive chemotactic responses to plant constituents of limited botanical distribution. A more recent definition of this term (9) appears less convenient.

Much of the literature on chemotactic responses in insects is not clearly relevant to the problem of host specificity in phytophagous insects. Among the relatively few studies of the effect of specific chemicals in plants on host plant selection in insects we may include those of Grevillius (16), Verschaffelt (29), Raucourt and Trouvelot (22), Dethier (6, 7, 8), Chauvin (3), Thorpe *et al.* (25), and Kuhn and Gauhe (20). Studies of chemoreception in leaf-eating insects have been conducted in which the substances tested have scarcely any possible relation to chemical constituents of plants that might influence host selection. When the object is to study the mechanism of chemoreception in terms of physicochemical principles as in the work of Frings (13) this is justified. Eger (11) studied the responses of caterpillars to substances representative of the four taste modalities of the human sense. His attempt to interpret his findings in terms of the role of gustation in leaf eating insects is highly questionable. Reviews of the general subject of chemoreception in insects have recently appeared (9, 10). The older literature is reviewed by several authors (2, 14, 19, 21, 27).

A classic example of oligophagy is afforded by the insects specific to cruciferous plants. These include the cabbage butterflies, *Pieris rapae* (L.) and *P. brassicae* (L.) and the diamondback moth, *Plutella maculipennis* (Curt.) The tissues of the cruciferous plants characteristically contain one or more

of a group of substances called the mustard oils or alkyl isothiocyanates (23). These generally occur in the form of glucosides. The same plants produce a specific enzyme, myrosin, which can hydrolyze mustard oil glucosides. This enzyme is contained in different cells than the glucosides and does not normally come into contact with them unless the plant tissue is bruised.

The best known of these glucosides is sinigrin or potassium myronate found in *Brassica nigra* Koch which contains allyl isothiocyanate, glucose, and potassium acid sulphate. Another glucoside, sinalbin, which occurs in *Brassica alba* Boiss., has a more complex structure and contains *p*-hydroxybenzylisothiocyanate, choline, and sinapinic acid. The taste of these glycosides to the human sense is bitter, whereas their mustard oils have a strong burning taste and a pungent odor. According to Schneider (23), another glucoside, glucocheirolin, found in *Cheiranthus cheiri* L., is tasteless and its mustard oil, cheirolin, is tasteless and odorless to humans.

The significance of a mustard oil glucoside in the choice of food plants by an insect specific to Cruciferae was first noticed by Verschaffelt (29). Using *Pieris rapae* (L.) and *P. brassicae* (L.) he first offered to the larvae the leaves of a great variety of plants of a number of different families. He found that the leaves of Cruciferae were accepted as well as those of some Resedaceae, Tropaeolaceae, Moringaceae, and Capparidaceae which also contain mustard oil glucosides. The leaves of other plants were refused. Next he painted a solution of sinigrin on the leaves of plants which had been rejected and found that the larvae now readily attacked some of these. He attributed failures to respond to this treatment to the presence of substances which repelled the larvae or masked the effect of the sinigrin. He obtained the same response when he painted the juice of *Bunias orientalis* L., a crucifer, on rejected leaves. The larvae could also be induced to eat flour, starch, and filter paper if these were treated with crucifer juice. Since hydrolysis would occur in the expressed juice, he concluded that the larvae respond to the taste of the fission products as well as to the unsplit glucosides. He suggested that sinigrin itself is perhaps not attractive but is perhaps hydrolyzed in the mouth of the larva. He did not, however, test this hypothesis experimentally.

The work of Verschaffelt was taken as a starting point for the present study. The response of an insect specific to Cruciferae and other plant families containing mustard oils and their glucosides lends itself to experimental study since much of the chemistry of these compounds is known. They are classified and described by Van Rijn and Dieterle (28), Armstrong and Armstrong (1), and by Schneider (23). Wehmer (31) has outlined their botanical distribution. The study was further facilitated by the availability of a favored host plant, the cabbage, throughout the year. As *Pieris* spp. are less convenient laboratory animals, the diamondback moth, *Plutella maculipennis* (Curt.), was used for the greater part of the work. It has similar host plant preferences, is easier to rear, and has no diapause.

Methods

The simple technique of Verschaffelt (29) was used in some of the preliminary experiments. This method requires that living test plants be available, which limits experimentation during the winter. It does not lend itself to quantitative determination of feeding responses to media treated with various mixtures of test substances at different concentrations nor does it provide for experimental control of physical characteristics of the media. The following sections describe the techniques evolved to overcome these difficulties.

Treatment of Plant Material

The freshly picked leaves of test plants were placed in a single layer in copper mesh trays and treated with steam for five minutes in order to kill enzymes quickly. They were then dried in a current of air at 60 to 70° C. for two or three hours. Adequate blanching in steam and the avoidance of excessive temperatures during the subsequent drying was essential to the preparation of good samples of dried leaves. The preservation of fresh green color was adopted as the criterion of proper treatment. The dehydrated leaves were packed in kilner jars and kept in dark cupboards. When needed for experiments the dried leaves were pulverized and sieved.

Preparation of Experimental Diets

A desirable basis for a test diet should resemble leaves in physical characteristics and lack a repellent taste or odor. The use of agar fulfilled these requirements. Each diet consisted of the test substances incorporated in 50 ml. of 3% agar. The mixture was heated to 85° C. and allowed to cool to 55° C. and poured into Petri plates of 9 cm. diameter. Snugly fitting circles of filter paper were placed in the outer plates. Usually five plates of each diet were poured. Five similar larvae of the third or early fourth instar were placed on each plate and kept at 21° C. overnight. The plates were kept inverted so that practically all the frass pellets dropped on the filter paper where they were easy to count.

Estimation of Feeding Responses

At the end of the test feeding period, the number of live larvae on each plate was noted as occasionally one or more died owing to disease, injury, cannibalism, or poisonous effect of some constituent in the diet. Since larval losses were relatively infrequent and their effect on the counts could not be estimated these data are not presented in the tables. As all the experiments were repeated at least once there was little possibility that this source of error might lead to misinterpretation.

Since a direct estimate of the feeding response by weight was not practicable the counts of frass pellets were taken to be a measure of the attractiveness of the diets. This involved two sources of error for which neither experimental nor statistical control was feasible. The total excreta depend on utilization as well as on the weight of food ingested. Also the average weight of frass

pellets might vary with different diets. The results obtained have been interpreted on the assumption that these effects are relatively inappreciable. Statistical criteria were based on the use of the tables provided by Fisher and Yates (12).

Chemical Methods

Sinigrin was prepared by a modification of the method of Hérissey and Boivin (18). It was found necessary to decolorize the black mustard seed extracts in order to obtain crystallization. The preparation of sinalbin was based on the method described by Van Rijn and Dieterle (28). It was found that purification could be hastened by passing the white mustard seed extracts through a column of alumina and by elution with glacial acetic acid from which crystallization occurred readily. As a check on the identity and purity of the sinigrin and sinalbin preparations, samples were submitted to Dr. G. Weiler and Dr. F. B. Strauss, Microanalytical Laboratory, Oxford, for sulphur and nitrogen analyses. The percentage values reported by this firm agreed closely with the theoretical values for both glucosides as shown in the following table.

	Sinigrin		Sinalbin	
	Sulphur	Nitrogen	Sulphur	Nitrogen
Calculated	15.44	3.37	7.78	3.40
Analysis	15.59	3.13	7.37	3.25

Myrosin was prepared by the method of Heiduschka and Pyriki (17). The preparation of glucocheirolin, as outlined by Schneider (23), was attempted but a crystalline product was not obtained. Allyl isothiocyanate was prepared by steam distillation of black mustard flour macerate. The estimation of mustard oil in steam distillates is that based on the Volhard titration described by Stahmann *et al.* (24).

Results

Feeding Responses to Fresh Leaves

As a first step, experiments of the type carried out by Verschaffelt (29) were repeated, but *Plutella maculipennis* (Curt.) as well as *Pieris brassicae* (L.) were used. Also, the glucoside sinalbin was tested as well as sinigrin. *P. brassicae* readily ate the leaves of 16 common species of Cruciferae as well as *Tropaeolum majus* L., *Reseda odorata* L., and *R. luteola* L., all of which are known to contain mustard oils.

Twenty-one species of plants that do not contain mustard oils were offered to *P. brassicae* larvae in three tests. In one, the leaves were painted with 3% sinigrin, in another with 3% sinalbin, and in the third the leaves were untreated. Some of the tests were repeated two or three times. None of the

untreated leaves were chewed perceptibly. The larvae were induced to feed at least slightly on seven of the plant species when the leaves were treated with a mustard oil glucoside. Feeding responses to these plants are shown in Table I. This experiment was repeated with 20 replicates with onion leaves. Only one of the untreated onion leaves was eaten while 18 of those treated with sinigrin and 17 treated with sinalbin were eaten. Onions contain sulphur oils other than mustard oils but these are evidently not attractive nor repellent.

TABLE I

RELATIVE PALATABILITY TO *P. brassicae* LARVAE OF LEAVES OF PLANTS TREATED WITH 3% MUSTARD OIL GLUCOSIDE SOLUTIONS

Plant	Untreated	Sinigrin	Sinalbin
Onion *Allium cepa* Linn.	−	+++++	++++
Marigold *Calendula* sp.	−, −	+, −	−, −
Cucumber *Cucumis sativus* Linn.	−	+++++	+++
Lettuce *Lactuca sativa* Linn.	−, −	−, −	+, −
Common malva *Malva sylvestris* Linn.	−, −	+++, ++	−, −
Poppy *Papaver* sp.	−, −	+++, −	−, −
Pea *Pisum sativum* Linn.	−, −, −	++, ++, +++	++, ++, −

NOTE: *The number of plus signs is proportionate to the amount of leaves eaten. Results of replicate tests are separated by commas.*

Attempts to induce *P. brassicae* larvae to feed on cucumber leaves treated with mustard oil solutions were unsuccessful. Since treatment with either sinigrin or sinalbin renders this plant attractive the unhydrolyzed glucoside and not the aglucone, mustard oil, is the effective feeding stimulant, as was confirmed in other experiments described below.

Forty-one species of plants reported to contain mustard oils were offered to *P. maculipennis* larvae. These are listed in Table II. All of these were accepted more or less readily by the larvae except *Carica papaya* Linn. which was refused and *Reseda alba* Linn. was eaten only slightly. As the leaves of *C. papaya* are very tough it is probable that the larvae were unable to chew them. The *Brassica* spp. were eaten more avidly than the other species and it is possible that the latter may contain slightly repellent substances as well as feeding stimulants.

TABLE II

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO LEAVES OF PLANTS THAT CONTAIN MUSTARD OILS

Cruciferae		Cruciferae—*Concluded*	
Alliaria officinalis Reichb.	+++	*Lunaria rediviva* L.	++, ++
Alyssum incanum	++, +	*Matthiola incana* R. Br.	++++, ++
Alyssum spinosum roseum	++, ++	*Pethionema pulchellum*	++, +++
Arabis caucasica	+++, +++	*Raphanus raphanistrum* L.	+++
Aubretia deltoides	−, ++	*Sisymbrium irio* L.	+++, +++
Barbarea verna Achers.	+++	*Sisymbrium strictissimum* L.	+++, +++
Barbarea vulgaris R. Br.	++, +	*Sisymbrium thallianum* J. Gay	++
Brassica alba Boiss.	++++	*Thlaspi arvense* Linn.	+++, +++
Brassica napus	+++++		
Brassica nigra Koch	+++++	Caricaceae	
Brassica oleracea L.	+++++	*Carica papaya*	−, −
Brassica rapa	+++++		
Brassica sinapis Visiana	+++	Capparidaceae	
Bunias orientalis L.	+++, +++	*Capparis flexuosa*	+++, ++++
Capsella bursa-pastoris Medic.	++++	*Capparis spinosa*	++++, ++++
Cardamine hirsuta Crantz	++++		
Cardamine pratensis	+++	Moringaceae	
Cheiranthus cheiri L.	+++	*Moringa ptygosperma*	+++, +++
Crambe cordifolia Steven	+++, +++		
Crambe orientalis L.	+++, +++	Salvadoraceae	
Draba verna Linn.	+++	*Salvadora persica*	+++, −
Iberis sempervirens	++, ++		
Isatis alpina	++, ++	Resedaceae	
Isatis tinctoria L.	++, ++	*Reseda alba* L.	+, −
Lepidium draba L.	++++		
Lepidium graminifolium L.	+, +++		

NOTE: *The number of plus signs is proportionate to the amount of leaves eaten. Results of replicate tests are separated by commas.*

Fourteen species of plants that do not contain mustard oils were offered to *P. maculipennis* larvae in three tests in which treatment with 3% sinigrin and sinalbin solutions were compared with responses to the untreated leaves. Positive feeding responses to seven of these species were obtained and these are presented in Table III. *S. vulgaris* and *V. angustifolia* were eaten slightly

TABLE III

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO LEAVES OF PLANTS TREATED WITH 3% SINIGRIN OR SINALBIN

Plant	Untreated	Sinigrin	Sinalbin
Lamium album	−, −	−, −	+, −
Malva sylvestris	−	+	++
Plantago major	−	+	−
Pisum sativum	−	+++	+++
Senecio vulgaris	++, −, −	+, −, +	+++, −, −
Stellaria media	−, −	−, −	+, −
Vicia angustifolia	+, −	−, −	−, +

NOTE: *The number of plus signs is proportionate to the amount of leaves eaten. Results of replicate tests are separated by commas.*

without treatment with glucosides. *P. sativum* appears to be more palatable than the other species when treated with either sinigrin or sinalbin. In another experiment, pea leaves treated with 3% sinigrin were compared with untreated pea leaves in 20 replicates. Positive feeding responses were obtained with 8 of the untreated leaves while 13 of the treated leaves were eaten. Apparently pea leaves contain no substances that are appreciably repellent to *P. maculipennis* larvae so that some feeding may occur and when sinigrin is added the larvae feed more readily.

Feeding Responses to Leaf Powder Gels

In a series of experiments a study was made of the feeding responses of *P. maculipennis* larvae to agar diets containing leaf powder preparations of various host plants. The object of this group of preliminary tests was to obtain information concerning the general characteristics of agar gel media containing powdered dehydrated leaves and other constituents and the suitability of such preparations for experimental study of feeding responses of the larvae to specific chemical constituents.

Effect of Concentration of Total Nutrients

Feeding responses of diamondback moth larvae to various concentrations of leaf powders of white mustard, black mustard, and rape in 3% agar gel were tested. The results obtained in one test of white mustard leaf powder presented in Table IV will serve to illustrate the typical response to concentrations of total nutrients (including feeding stimulants). A clear-cut correlation between feeding response and concentration of total nutrients in the range 2–8% is obtained. Since all the nutrients change in concentration it cannot be assumed that the response is governed only by the concentration of a specific feeding stimulant. This distinction has been overlooked by earlier workers (26). That the effectiveness of a feeding stimulant may be markedly affected by the presence of other (nutrient) constituents was shown by a preliminary experiment in which sinigrin did not render agar attractive in the absence of nutrients. As will be shown later, agar media containing nutrients which are themselves only moderately attractive become highly palatable on the addition of sinigrin.

TABLE IV

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO CONCENTRATIONS OF WHITE MUSTARD LEAF POWDER IN 3% AGAR GEL

Diet No.	% Leaf powder	Mean frass count	Standard error
1	2.0	178.0	15.0
2	4.0	280.3	50.1
3	6.0	498.7	35.0
4	8.0	549.7	40.6
5	10.0	494.7	66.5

NOTE: *Number of replicates = 3.*

Feeding Responses to Aqueous Extracts

An aqueous extract of rape leaf powder was tested in a series of dilutions. As in tests with different concentrations of whole white mustard leaf powder a clear-cut positive regression of feeding response on concentration was obtained. A comparison was made of feeding response to whole leaf powder, the aqueous extract, and the extracted residue of white mustard leaf powder. The feeding responses as measured by frass counts are shown in Table V which also shows a typical negative response to agar gel containing no nutrients. Although no attempt was made to extract exhaustively the residue tested in Diet 4 it is scarcely more attractive than pure agar alone. It is curious that water extracts practically all the feeding stimulant from the leaf powder and yet the aqueous extract is itself not as attractive as the whole leaf powder. This bears out what was said in the foregoing subsection concerning the dependence of sinigrin on the presence of other substances for the full expression of its stimulating power. It is possible that texture of the medium is of some importance in the attractiveness of a medium but no evidence was obtained to support this view.

TABLE V

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO AQUEOUS EXTRACT AND RESIDUE OF WHITE MUSTARD LEAF POWDER IN 3% AGAR GEL

Diets	Mean frass count	Standard error
1. Agar only (no leaf powder)	23.0	5.0
2. White mustard leaf powder, 1.0%	167.5	14.5
3. 50 ml. aqueous extract of white mustard leaf powder	88.5	10.7
4. Residue of (3)	42.0	5.5

NOTE: *Number of replicates = 4.*

TABLE VI

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO WHITE MUSTARD LEAF POWDER IN 3% AGAR GEL BUFFERED AT VARIOUS HYDROGEN ION CONCENTRATIONS

Diet No.	pH	Mean frass count	Standard error
1	4.5	322.0	54.9
2	5.9	335.0	117.4
3	6.6	294.3	19.9
4	7.6	247.7	34.7
5	8.3	225.0	31.4

NOTE: *Number of replicates = 3.*

Effect of Hydrogen Ion Concentration

White mustard leaf powder at various hydrogen ion concentrations was tested. The pH values given refer to the reaction of the water used to make up the diets. Sorensen's buffer mixtures (15, p. 85) were used to adjust the pH. The feeding responses to the buffered diets are given in Table VI. Although the means do not differ significantly the feeding responses fall off with increasing alkalinity and the highest response is obtained at pH 5.9. It is of interest that this value is not greatly different from that of aqueous extracts of black mustard and white mustard leaf powder which were found in duplicate determinations to be 6.1 and 6.3 respectively.

Feeding Responses to Leaf Powders of Acceptable Host Plants

Feeding responses to agar media containing leaf powder preparations of several species of Cruciferae were tested. As in tests with fresh leaves the *Brassica* species appear to be most attractive. For this reason leaf powder gels of white and black mustard and rape were used as controls in the experiments described hereafter. Cabbage leaf powder is also highly attractive but good samples are more difficult to prepare as the leaves are thick. Cabbage leaf powder in agar gel was compared with circles of fresh cabbage leaf cut to fit the Petri plates. The mean frass counts of five replicates for fresh cabbage leaf was 503.4 and for cabbage leaf powder, 487.8. The mean difference was not significant so that carefully prepared leaf powder media appear to be substantially as attractive as the fresh leaves.

Feeding Responses to Leaf Powder Gels of Unacceptable Plants

It has been shown that *P. maculipennis* larvae do not feed on the fresh leaves of plants that do not contain mustard oil glucosides. The same negative response was obtained in tests of leaf powder diets prepared from such plant species. The effect of treating several of these preparations with mustard oil glucosides was studied.

Feeding Responses to Leaf Powder Gels and Mustard Oil Glucosides

The effect of treating leaf powder gels of beet, tomato, cucumber, and red clover with sinigrin was tested in a series of experiments. The results obtained with tomato leaf powder and sinigrin are typical and these are presented in Table VII. This table also demonstrates that tomato contains a repellent since its aqueous extract depresses the feeding response to black mustard. The other plants tested probably also contain repellents since the addition of sinigrin did not induce the larvae to feed on them. However, the aqueous extract of cucumber did not depress the response to black mustard. Perhaps cucumber contains some repellent substance which is not extracted with water.

It will be recalled that pea leaves treated with sinigrin were more readily accepted by *P. maculipennis* larvae than other nonhost plants. A preliminary test showed that pea leaf powder agar gel with 0.2% sinigrin is substantially

TABLE VII

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO TOMATO LEAF POWDER IN 3% AGAR GEL TREATED WITH 0.01% SINIGRIN AND TO BLACK MUSTARD LEAF POWDER GEL CONTAINING AQUEOUS EXTRACT OF TOMATO LEAF POWDER

Diets	Mean frass count	Standard error
1. Agar only (no leaf powder)	25.3	5.0
2. Tomato leaf powder, 1.0%	32.0	15.9
3. (2) + sinigrin, 0.01%	47.3	5.8
4. Black mustard leaf powder, 1.0%	146.3	24.8
5. (4) + aqueous extract of tomato leaf powder	39.6	6.6

NOTE: *Number of replicates = 3.*

as attractive to *P. maculipennis* larvae as mustard leaf powder gel. As pea leaf powder was the only preparation that provided satisfactory feeding responses on treatment with sinigrin, it was used for the further study of feeding reponses to specific substances.

FEEDING RESPONSES TO MUSTARD OIL GLUCOSIDES

The influence of the mustard oil glucosides, sinigrin, sinalbin, and glucocheirolin on *P. maculipennis* larvae was tested on pea leaf powder agar gels containing these substances.

Feeding Responses to Sinigrin

P. maculipennis larvae were tested on pea leaf powder gels containing a series of concentrations of sinigrin. The feeding responses are shown in Table VIII. The responses to sinigrin are highly significant when compared

TABLE VIII

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO A SERIES OF CONCENTRATIONS OF SINIGRIN IN 3% AGAR GEL CONTAINING 2.0% PEA LEAF POWDER

Diets	Mean frass count	Standard error
1. Pea leaf powder, 2.0%	17.8	1.2
2. (1) + sinigrin, 1000 p.p.m.	208.2	24.3
3. (1) + sinigrin, 500 p.p.m.	158.0	53.8
4. (1) + sinigrin, 250 p.p.m.	166.0	27.7
5. (1) + sinigrin, 125 p.p.m.	160.0	23.4
6. (1) + sinigrin, 62.5 p.p.m.	176.6	24.4
7. (1) + sinigrin, 31.2 p.p.m.	146.0	25.8
8. (1) + sinigrin, 15.6 p.p.m.	176.0	32.7
9. (1) + sinigrin, 7.8 p.p.m.	167.2	32.3
10. Black mustard leaf powder, 2.0%	171.6	46.4

NOTE: *Number of replicates = 5.*
z = 1.3.
L.S.D. (5.0%) = 67.4

with the control, Diet 1. The response to Diet 9 indicates that the threshold concentration of sinigrin is at least as low as 8 p.p.m. On the logarithmic scale* proposed by Thorpe *et al.* (25) this corresponds to a value of more than five. Again, pea leaf powder treated with sinigrin is as attractive as black mustard leaf powder.

Feeding Responses to Sinalbin

In a preliminary experiment it was found that pea leaf powder gel treated with sinalbin yielded feeding responses comparable to those with sinigrin. In a single experiment the effects of sinigrin and sinalbin were compared in parallel concentrations. The results are presented in Table IX. The lowest concentration eliciting a response to sinigrin is 2 p.p.m. This corresponds to an activity of nearly six on the logarithmic scale. The sinalbin concentration of 20 p.p.m. in Diet 9 excites a feeding response that just fails to attain significance. Since this concentration is probably very near the threshold we may say that the activity of sinalbin is nearly five logarithmic units. On the whole, sinigrin is more attractive than sinalbin. This superiority cannot be accounted for by a correction for molecular weight.

TABLE IX

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO CONCENTRATIONS OF SINIGRIN AND SINALBIN IN PEA LEAF POWDER GELS

Diets	Mean frass count	Standard error
1. Pea leaf powder, 2.0%	40.0	5.9
2. (1) + sinigrin, 2000 p.p.m.	217.2	21.4
3. (1) + sinigrin, 200 p.p.m.	183.2	16.7
4. (1) + sinigrin, 20 p.p.m.	108.0	20.9
5. (1) + sinigrin, 2 p.p.m.	81.4	15.7
6. (1) + sinigrin, 0.2 p.p.m.	68.4	7.5
7. (1) + sinalbin, 2000 p.p.m.	121.4	26.1
8. (1) + sinalbin, 200 p.p.m.	118.4	21.5
9. (1) + sinalbin, 20 p.p.m.	71.2	20.8
10. (1) + sinalbin, 2 p.p.m.	68.0	13.2
11. (1) + sinalbin, 0.2 p.p.m.	61.4	7.1

NOTE: *Number of replicates = 5.*
z = 1.2.
L.S.D. (5.0%) = 31.4.

Feeding Responses to Glucocheirolin

P. maculipennis larvae were tested on pea leaf powder containing glucocheirolin. The feeding responses to this preparation are shown in Table X. Since this glucoside was not prepared in crystalline form it was probably impure and the concentrations are unknown except in terms of dilutions of the stock solution. However, it is unlikely that the observed stimulation of

** The unit of this scale is log x to base 10, where x is the number of ml. of water, containing 1 gm. of dissolved substance required to reach the threshold.*

feeding is due to impurities in the glucocheirolin preparation but we cannot be certain of this. Since the variance ratio is highly significant the response to the glucocheirolin preparation is real but does not differ for the three concentrations.

TABLE X

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO GLUCOCHEIROLIN IN PEA LEAF POWDER GELS

Diets	Mean frass count	Standard error
1. Pea leaf powder, 2.0%	47.8	9.1
1. (1) + stock glucocheirolin solution	131.8	7.9
3. (1) + $\frac{1}{2}$ dilution of stock glucocheirolin solution	126.8	33.4
4. (1) + $\frac{1}{4}$ dilution of stock glucocheirolin solution	142.6	11.2
5. Wallflower leaf powder, 2.0%	179.2	13.0
6. Rape leaf powder, 2.0%	203.0	19.5

NOTE: *Number of replicates* = 5.
$z = 1.0$.
L.S.D. (5.0%) = 39.3.

EFFECT OF THE ENZYME, MYROSIN, ON FEEDING RESPONSES

The foregoing experiments show that nutrient mixtures that contain mustard oil glucosides but no mustard oil nor enzyme are highly palatable to *P. maculipennis* larvae. Therefore the frequently stated hypothesis that the mustard oils in free form are the real feeding stimulants is highly improbable. This question was further investigated in experiments wherein the feeding responses to leaf powders of host plants treated with a preparation of the enzyme myrosin were tested. This enzyme hydrolyzes the glucosides in the leaf powders. If mustard oil is the active principle these diets should be at least as attractive as the untreated controls.

The effect of treatment with myrosin was studied in a series of 17 preliminary experiments wherein the effect of adding myrosin to turnip, black mustard, nasturtium, wallflower, and horse-radish leaf powder was tested. The results were very variable but in none of these experiments did myrosin treatment increase the feeding responses. In 11 of the experiments the feeding responses were reduced but only two of these were statistically significant.

Since these tests were on a small scale the results were pooled in order to apply a test of significance on a larger body of data. This is legitimate since there is a logical basis for applying a "*t*" test to differences of pairs of observations within replicates. The mean difference was 25.1 ± 11.5. The value of "*t*" was 2.17 and the 5.0% point 1.99. This supports the contention that myrosin tends to decrease the palatability of leaf powder preparations of host plants. The inference appears to be that the mustard oil glucosides are more attractive than their fission products.

An interesting result was obtained with nasturtium leaf powder treated with myrosin. In this experiment, most of the larvae died. This suggests that the mustard oil released by the hydrolysis was in lethal concentration.

In some of the experiments myrosin denatured by immersion in boiling water was tested. In none of these tests did denatured myrosin have any effect on the attractiveness of the diets. Apparently the depressing effect of myrosin on feeding responses is due to its enzymatic properties and it has no direct repellent effect.

An attempt was made to determine whether time and temperature of the enzyme reaction influence feeding responses to black mustard leaf powder agar gel containing myrosin. The diets tested and the results obtained are shown in Table XI. The enzyme reactions were stopped at the indicated times by immersion of the vessels in boiling water. Although none of the diets containing myrosin elicited as high a feeding response as did the control diet the mean differences are clearly significant only for Diets 2 and 3.

No detailed interpretation seems feasible for these data unless we assume that the mustard oil is rapidly released in repellent concentration at 35–70° C. but is lost by evaporation at the longer time intervals, leaving sufficient unhydrolyzed sinigrin at the equilibrium concentration to stimulate feeding. The potassium acid sulphate released by the hydrolysis would inhibit continued fission of the sinigrin (1). In any case the results do not lend support to the view that mustard oil is the principal stimulus to feeding. This was further confirmed in the experiments described below.

TABLE XI

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO BLACK MUSTARD LEAF POWDER DIETS TREATED WITH MYROSIN FOR VARIOUS TIME INTERVALS

Diet	Conditions of enzyme action		Mean frass count	Standard error
	Temp., °C.	Time, min.		
1. Basic diet* + denatured myrosin, 0.2%			407.2	23.6
2. " " + myrosin, 0.2%	70	10	147.6	46.3
3. " " + " 0.2%	35	1	234.2	37.6
4. " " + " 0.2%	35	5	345.6	40.5
5. " " + " 0.2%	35	10	355.0	28.6
6. " " + " 0.2%	35	15	310.6	27.3
7. " " + " 0.2%	35	20	350.6	31.6
8. " " + " 0.2%	35	30	376.8	41.9

* *Basic diet consists of 2.0% black mustard leaf powder in 3% agar.*

NOTE: *Number of replicates = 5.*

$s = 0.86.$

L.S.D. $(5.0\%) = 73.7.$

FEEDING RESPONSES TO ALLYL MUSTARD OIL

Since the hydrolysis of mustard oil glucosides in leaf powder gels is complex and the relative concentrations of glucosides and fission products could not conveniently be determined, it was decided to isolate the mustard oil of

sinigrin and test the feeding responses to this preparation alone and in combination with sinigrin at various concentrations of each. The object of this was to simulate some of the conditions that may obtain at several stages in the hydrolysis of sinigrin with the advantage of having some knowledge as to the concentrations concerned.

An aqueous solution of allyl mustard oil was prepared in the form of the steam distillate from the hydrolysis of the sinigrin contained in 10 gm. of black mustard flour. The solubility of allyl mustard oil in water is 0.2%. The concentrations obtained in the steam distillates were below 0.1%. In preliminary tests with synthetic allyl mustard oil, saturated solutions were repellent and lethal to the larvae. The lower concentrations obtained in the steam distillates were not lethal. Where mustard oil determinations were made on the steam distillates the concentrations are stated. Otherwise the concentrations are known only in terms of dilutions of the stock distillate.

Effect of Mustard Oil in the Absence of Sinigrin

Feeding responses of *P. maculipennis* larvae were tested on pea leaf powder gels made up with dilutions of a steam distillate containing allyl mustard oil. The results are shown in Table XII. None of the apparent responses to the steam distillate is significant. On the other hand the response to sinigrin is striking. It is evident that mustard oil in the absence of sinigrin has little potency if any as a feeding stimulant, whereas sinigrin alone is highly potent. It remains to be determined whether interactions appear (as manifested in feeding responses) when pea leaf powder gels contain mixtures of sinigrin and allyl mustard oil in various concentrations.

TABLE XII

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO PEA LEAF POWDER GELS CONTAINING DILUTIONS OF STEAM DISTILLATE CONTAINING ALLYL MUSTARD OIL

Diets	Mean frass count	Standard error
1. Pea leaf powder, 2.0%	42.4	8.8
2. (1) + stock solution of steam distillate	41.2	16.3
3. (1) + $\frac{1}{2}$ dilution of stock distillate	76.0	24.4
4. (1) + $\frac{1}{4}$ dilution of stock distillate	75.2	18.7
5. (1) + $\frac{1}{8}$ dilution of stock distillate	70.2	9.6
6. Pea leaf powder, 2.0% + sinigrin, 0.02%	236.0	34.8

NOTE: *Number of replicates* = 5.
$z = 2.6$.
L.S.D. (5.0%) = 41.4.

Effect of Mixtures of Mustard Oil and Sinigrin

Constant Sinigrin and Varying Mustard Oil Concentrations

Pea leaf powder gels were made up containing equal concentrations of sinigrin but different mustard oil contents. The allyl isothiocyanate in the stock steam distillate was determined and the concentrations in the dilutions

were calculated from this. Feeding responses of *P. maculipennis* larvae to these mixtures are presented in Table XIII. All the responses to diets containing sinigrin with or without mustard oil are significant but the response to mustard oil alone (Diet 6) is not.

This experiment was repeated on a still larger scale with similar results except that the diets containing 9/16 and 27/64 dilutions of the stock mustard oil preparation with sinigrin in pea leaf powder gel excited significantly greater feeding responses than the other media which contained other concentrations of mustard oil or lacked mustard oil. Unfortunately no mustard oil determinations were made on the distillate. However a similar result was obtained later (Table XIV, Diet 4) where the concentrations of sinigrin and mustard oil are known.

TABLE XIII

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO PEA LEAF POWDER GELS MADE UP WITH MIXTURES OF ONE CONCENTRATION OF SINIGRIN WITH VARIOUS DILUTIONS OF STEAM DISTILLATE CONTAINING MUSTARD OIL

Diets	Mean frass count	Standard error
1. Pea leaf powder, 2.0%	37.0	4.5
2. (1) + sinigrin, 0.05%	199.2	23.5
3. (2) + stock steam distillate, 0.0237%	199.6	34.8
4. (2) + dilution steam distillate, 0.0059%	188.4	18.3
5. (2) + dilution steam distillate, 0.00148%	231.6	34.4
6. (1) + stock steam distillate, 0.0237%	63.8	15.1
7. Cabbage leaf powder, 2.0%	134.4	29.8
8. (7) + stock steam distillate, 0.0237%	160.2	28.5

NOTE: *Number of replicates = 5.*
z = 1.0.
L.S.D. (5.0%) = 53.0.

Constant Mustard Oil and Varying Sinigrin Concentration

An experiment in which pea leaf powder gels containing a constant concentration of allyl mustard oil (980 p.p.m.) and a series of concentrations of sinigrin (0.64–2000 p.p.m.) were tested provided interesting data which are presented in Table XIV. The response to Diet 9 is significantly greater than that to Diet 3 which shows that sinigrin in the presence of 0.098% allyl mustard oil is palatable at a concentration of less than 1 p.p.m. This corresponds to an activity of over six on the logarithmic scale. An activity approaching this value, however, was obtained in a previous experiment (Table IX, Diet 5) in the absence of mustard oil so that it is not certain whether the threshold concentrations for sinigrin are lower in the presence of mustard oil. The mustard oil alone (Diet 3) does not enhance the palatability of pea leaf powder gel. However, a comparison of the responses to Diets 2 and 4 shows that mustard oil at this concentration increases the palatability of pea leaf powder gel containing 2000 p.p.m. sinigrin.

TABLE XIV

FEEDING RESPONSES OF *P. maculipennis* LARVAE TO PEA LEAF POWDER AGAR GELS CONTAINING MIXTURES OF 0.098% MUSTARD OIL WITH A SERIES OF CONCENTRATIONS OF SINIGRIN

Diets	Mean frass count	Standard error
1. Pea leaf powder, 2.0%	41.2	4.4
2. (1) + sinigrin, 0.2%	133.0	20.1
3. (1) + steam distillate	41.2	15.0
4. (3) + sinigrin, 0.2%	181.8	29.4
5. (3) + sinigrin, 0.04%	138.0	20.9
6. (3) + sinigrin, 0.008%	126.6	22.3
7. (3) + sinigrin, 0.0016%	95.6	12.4
8. (3) + sinigrin, 0.00032%	114.8	12.8
9. (3) + sinigrin, 0.000064%	80.6	16.0
10. Cabbage leaf powder, 2.0%	135.4	14.3

NOTE: *Number of replicates* = 5.
$z = 1.2$.
L.S.D. (5.0%) = 26.7.

This experiment was repeated with a greater range of sinigrin concentrations. The results were very similar except that significant feeding responses to sinigrin in the presence of steam distillate containing mustard oil were not obtained at concentrations below 31 p.p.m. This threshold concentration is somewhat higher than was obtained in other experiments but there is little basis for interpretation of differences of this order in comparisons of results from different experiments.

While these results do not satisfactorily explain the results obtained in the myrosin experiments (Table XI), they suggest that investigations along these lines coupled with chemical studies of the course of hydrolysis of sinigrin in leaf powder mixtures may clarify the precise relationship between sinigrin and its fission products in so far as they affect the chemotactic feeding responses of *P. maculipennis* larvae. No account has been taken of the possible chemotactic effect of another fission product of sinigrin, potassium acid sulphate. It is probable that this substance has an indirect effect by its inhibition of the enzyme myrosin. For the present, the data permit us to postulate that under certain conditions the hydrolysis of sinigrin may leave sufficient sinigrin to provide the principal feeding stimulus and release the optimum concentration of mustard oil required to enhance the palatability of the mixture.

OLFACTORY RESPONSES

A study was made of the olfactory responses of diamondback moth larvae to their host plants with a technique similar to that used by earlier workers (5). Although the results strongly indicate that the larvae can by olfaction perceive their host plants at short distances, control experiments to determine whether the stimulus is provided by humidity or to other vapors emanating

from the leaves were not completed. Since olfactory perception of leaves of food plants cannot be ignored the possible role of olfactory stimuli in the feeding behavior of *P. maculipennis* larvae must be considered.

Since mustard oil is an odoriferous substance, the increased feeding responses obtained in some experiments (Table XIV) where mustard oil is tested in the presence of sinigrin may be interpreted in terms of olfaction. If we assume that mustard oil elicits a *biting* response by stimulation of the olfactory sense of diamondback moth larvae then feeding will commence almost immediately when larvae are placed on nutrient media containing mustard oil at optimal concentrations. If mustard oil is not present then biting will be delayed until inanition forces the biting response by reducing the threshold concentration to zero. If the nutrient medium also contains sinigrin, feeding will continue, otherwise not. The increased feeding responses may be the result of more prompt commencement of feeding rather than a direct effect on palatability. The frequent failure of mustard oil to increase feeding responses to diets containing sinigrin may be attributed to variations in the concentration of this substance and to differences in the state of inanition of different batches of larvae.

Discussion

The Role of Mustard Oil Glucosides

It has been established that sinigrin which is a constituent of some of the host plants of *P. maculipennis* larvae furnishes a specific and potent stimulus which in the presence of suitable nutrients induces sustained feeding in this insect. Since the other two glucosides tested, sinalbin and glucocheirolin, also stimulate feeding, the mustard oil glucosides as a group, the distribution of which coincides with the range of acceptable host plants for *P. maculipennis*, are almost certainly responsible for the host plant specificity of this insect.

The sensitivity of gustatory perception in *P. maculipennis* for sinigrin is remarkable. The gustatory sense of insects has been compared unfavorably with that of humans (8). However, the writer was scarcely able to taste the most concentrated sinigrin solutions tested which were 1000 times as concentrated as the threshold concentrations of the larvae. Such comparisons are most illuminating when the test substances are of significance in the biology of the insect.

Feeding responses have been obtained in the Colorado potato beetle to purified preparations of a chemical constituent of its host plant (3). The diamondback moth does not respond in this way to sinigrin. Normal feeding responses were obtained only in media which contained leaf powder preparations or artificial mixtures of nutrients none of which are themselves appreciably palatable to the insect. While sinigrin is not a nutrient in the strict sense, its ability to stimulate feeding suggests an application in the development of an artificial medium for the study of nutrition in an oligophagous insect. Preliminary experiments in this direction were promising and will be described elsewhere.

The Role of Mustard Oil

Chemical studies of the course of hydrolysis of sinigrin by myrosin shed some light on the interpretation of the role of mustard oil in the feeding behavior of *P. maculipennis* larvae. As the fission product, potassium acid sulphate, is released it inhibits the activity of myrosin and brings the reaction to an equilibrium leaving some of the sinigrin unhydrolyzed (1). This explains the palatability of expressed juices of cruciferous plants. In view of the sensitivity of perception of sinigrin it is probable that the larvae can respond to the unhydrolyzed glucoside in such preparations.

The hydrolysis of sinigrin by myrosin requires a considerable period of time to reach equilibrium. Only a small fraction of the glucoside is split in one minute (24, Fig. 2). This is easily demonstrated by chewing the leaf of a plant such as black mustard. An appreciable time interval elapses before any taste of mustard oil is perceived. It is unlikely that morsels of ingested leaves remain in contact with the gustatory receptors of the feeding larvae long enough for much mustard oil to develop at the expense of the glucoside concentration. These observations lend further support to the contention that the mustard oil glucosides rather than their fission products are the principal plant constituents responsible for the acceptability of the host plants of *P. maculipennis* larvae.

Conclusion

These considerations support the postulate advanced by Dethier (5) for the probable sequence of events in the feeding of oligophagous larvae: (*a*) An olfactory stimulus aids the insect in finding food at short range and elicits a biting response. (*b*) A gustatory stimulus induces the insect to continue feeding. Behavioristic studies of the Colorado potato beetle led Chin (4) to a similar conclusion.

Summary

1. *Plutella maculipennis*, like *Pieris brassicae* and *Pieris rapae*, will feed on plants that contain mustard glucosides. They will feed on other plants if the leaves are painted with a solution of a mustard oil glucoside such as sinigrin or sinalbin if the leaves are not tough and do not contain repellents. Leaves treated with mustard oil were not eaten.

2. A medium adapted to the study of gustation in *P. maculipennis* larvae as expressed by feeding responses was developed. Leaves of plants were tested in the form of dry powders. These and other test substances were incorporated in agar gels. The amount of feeding was estimated by frass counts.

3. Experimental samples of sinigrin, sinalbin, glucocheirolin, allyl mustard oil, and myrosin were prepared from cruciferous seeds.

4. The concentration of total nutrients had a marked effect on the feeding responses of the larvae but hydrogen ion concentration does not appear to be critical.

5. The addition of sinigrin or sinalbin to pea leaf powder gel renders this medium highly attractive to the larvae. Glucocheirolin is also effective. Allyl mustard oil does not induce appreciable feeding unless the medium also contains a mustard oil glucoside when it moderately increases feeding at certain optimum concentrations.

6. The addition of viable myrosin to media containing sinigrin reduces feeding responses by depleting the quantity of glucoside. Heat-killed myrosin is not repellent nor does it otherwise inhibit feeding.

7. A probable sequence of stimulus response mechanisms that determine the behavior of *P. maculipennis* larvae when they find food is as follows: (*a*) an olfactory stimulus in the form of mustard oil induces prompt feeding, (*b*) if no mustard oil is present hunger presently induces biting responses, (*c*) if a feeding stimulant in the form of a mustard oil glucoside is present along with a supply of nutrients, normal feeding proceeds.

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